Changes in Ribonucleic Acids in Citrus Fruit With Growth and Development

By

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KEY TO SYMBOLS OF ABBREVIATIONS

AISR	Alcohol insoluble residue
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CMP	Cytidine monophosphate
CDP	Cytidine diphosphate
CTP	Cytidine triposphate
DNA	Deoxyribonucleic acid
IAA	Indole-3-acetic acid
GA	Gibberellic acid
GMP	Guanosine monophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
mCi	Millicurie
NAD^+	Nicotinamide-adenine dinucleotide
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate
RNA	Ribonucleic acid
mRNA	Messenger RNA
sRNA	Soluble RNA
tRNA	Transfer RNA
SDS	Sodium dodecyl sulfate
UMP	Uridine monophosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate

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CHANGES IN RIBONUCLEIC ACIDS IN CITRUS FRUIT WITH GROWTH AND DEVELOPMENT

By

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Quantitative and qualitative changes in RNA and acid-soluble nucleotides in the fruit pulp of 'Orlando' tangelo, and 'Hamlin', 'Pineapple' and 'Parson Brown' oranges during stages II and III of growth and development were studied. Analyses of these constituents were made with anion-exchange column chromatography, UV spectral analysis and gel-electrophoresis. It was concluded from this study that quantitative changes in RNA are gradual over the stages of fruit development examined. This conclusion was based on the following observations from total RNA and gel-electrophoretic determinations: Changes in concentration of total RNA were gradual from the mid-point of stage II until well into stage III, with only a slight increase just prior to completion of stage II of fruit growth. Gel-electrophoretic separation of RNA yielded patterns that indicated that RNA changes were gradual decreasing until the transition from stage II to stage III and then remaining fairly constant for at least 2 months, and possibly longer. In 'Calamondin' fruit, maximum incorporation of ^{32}P into RNA occurred also at the transition from stage II to stage III, and appreciable ^{32}P activity was found in the mRNA region of the electrophoretogram. It was suggested that the increased RNA in the early part of stage III of

fruit development may be essential for the initiation of changes associated with ripening of citrus fruit, e.g., production of enzymes required for changes normally associated with ripening.

Analysis of the acid-soluble nucleotide fraction of the fruit indicated that they are present in appreciable quantities in fruits and most surely play a vital role in fruit development. The nucleotides NAD, CMP, AMP, UMP, ADP, CTP, GDP, UDP, and ATP were tentatively identified based on the elution position from an anion-exchange column and spectral analysis. ATP was further assayed by the luciferin-luciferase method. The possible significance of nucleotides in carbohydrate metabolism and in the edible fruit was discussed, particularly as nucleotides may modify flavor.

INTRODUCTION

During growth and development of an organism an irreversible increase in size occurs, and there is an increase in net protein synthesis. In recent years, much work has been directed toward understanding this process. Yet, current physiological knowledge cannot adequately explain growth and development.

Numerous books and reviews have been written on the relation of nucleic acids to biological reactions. These indicate the importance attached to this group of compounds. Yet, very little is known of the relation of nucleic acids to the growth and development of a fruit.

Nucleic acids and their derivatives are of frequent occurrence in plant tissue. They are known to be essential for protein synthesis; storage and transfer of genetic information; and the mediation and utilization of respiratory energy for the synthesis of cellular components. An analysis of the changes of nucleic acids and their derivatives during growth and development should serve as an index of change in various biochemical processes. The actions of various compounds affecting growth and development have shown either a direct or indirect effect on nucleic acid metabolism (59). The mode of action of growth regulators has been suggested as being mediated through nucleic acid metabolism (57). Thus, an understanding of the changes of nucleic acids during this period could possibly contribute to the understanding of the regulatory mechanisms of growth and development and aid in the use of growth regulating compounds, particularly in altering fruit physiology.

This study is designed to investigate the quantitative and qualitative changes of RNA and acid-soluble nucleotides in the growth and development of several citrus varieties, including both seedy and non-seedy cultivars.

LITERATURE REVIEW

General Considerations

The citrus fruit is a modified berry called a hesperidum. For descriptive purposes, the fruit has been divided into 2 regions, the pericarp and the septa. The pericarp is that portion exterior to the carpels and is divided into 3 regions; the exocarp or flavedo, the mesocarp or albedo, and the endocarp, the inner portion of the pericarp and portion of the locular membrane. The edible portion of the fruit is the fused carpels containing the juice vesicles. The juice vesicles are multicellular structures originating from the locular wall, which are part of the endocarp, with a layer of cells on the surface that are small and epidermis-like with a cuticle covering. The internal cells are highly evacuolated (89).

Morphological and Anatomical Changes During Fruit Growth

Bain (6) reported that the growth of the 'Valencia' orange fruit is distinguished by 3 stages, each stage being characterized by distinct morphological, anatomical and physiological changes.

Stage I is the cell division stage since the number of cells in all of the tissues of the developing fruit increase to form the tissue of the mature fruit. Increase in fruit size during this stage is due primarily to growth of the pericarp. During this stage, the juice sac primordium grows by cell division and forms a club-shaped head supported by a stalk.

Stage II is the period of rapid increase in size, primarily by cell enlargement. This stage corresponds to the maximum growth period. There is a marked expansion of the tissues accompanied by cell enlargement and differentiation during this stage. The pulp tissue increases considerably, and the juice sacs continue to divide and enlarge accompanied by an increase in juice content. Cell division is completed in all tissues except the peel at the end of this stage.

Stage III is regarded as the period of maturation. This stage is readily distinguished from stage II by its decreased rate of morphological, anatomical, and physiological changes.

Physiological and Biochemical Changes

Nitrogenous compounds

In the mature citrus fruit, nitrogenous compounds compose 5 to 10% of the total solids. The primary nitrogenous compounds found in citrus fruits are amino acids, amines, peptides, and proteins (86).

Investigations into the changes of both catalytic and structural proteins during citrus fruit growth are limited. Bain (6) found that protein nitrogen increased greatly during the cell division stage in the fruit of the 'Valencia' orange, owing to the synthesis of new cytoplasm, but the total nitrogen content remained unchanged. Throughout stage II, protein contained the bulk of the nitrogen. During maturation, increase in protein nitrogen occurred at a decreased rate. The total protein concentration of the juice insolubles is reported to be approximately 34% at maturity (7).

Among the proteins in citrus fruits, major interest has been on enzymes, especially those that hydrolyze pectins. Pectinase was re-

ported to be associated with the insoluble components of the fruit and not present in the juice (51, 71). The occurrence of other enzymes such as acetylesterase (52), phosphatase (4), decarboxylase (5), peroxidase (30) and enzymes associated with mitochondria (23, 54, 105), have also been reported. In contrast to most enzyme systems, phosphatase activity was also found in the filtered juice.

A considerable number of amino acids have been identified as occurring in citrus fruit, and are found in all components of the fruit. During the early stages of fruit growth, ammonia, asparagine, aspartic acid, and serine were predominant. Ammonia declined to a negligible amount by maturity. Clements and Leland (28) found that in mature 'Valencia' fruits proline, arginine, and aminobutyric acid were the predominant amino acids. The amino acid nitrogen accounted for 61% of the total nitrogen in the young fruit and 83% at maturity.

Respiration

Respiration data on various types of fruits generally fall into one of two basic categories. Biale (12) has designated these as either climacteric fruits or non-climacteric fruits. In climacteric fruits the respiration declines throughout growth to a low level called the preclimacteric minimum, rises sharply to a climacteric maximum, and then drops markedly during the post-climacteric period. The sharp respiration increase is considered the point in the life of the fruit when growth and maturation are complete and the stage is set for senescence and deterioration (96). A concomitant increase in ethylene production also occurs during the climacteric (21).

Associated with the climacteric are a number of physical and chemical changes which are characteristic of the ripening process

such as color transformation, change in pectins from insoluble to soluble forms, and transformation of starches to sugars (13, 31, 46). These changes occur in fruits left on the tree and in harvested fruits, but the processes are accelerated with detachment if other conditions, particularly temperature, are similar (14, 41, 96).

The pattern of respiration of citrus fruit is decidedly different from climacteric fruit. Citrus fruits do not exhibit a pronounced rise in respiration and are classified as non-climacteric fruits. Bain (6) followed the metabolic activity in 'Valencia' oranges from early fruit set through the stages of cell division, enlargement and maturation. The respiration rate per fruit increased during the first 2 stages of growth but declined throughout the maturation stage. Ripening occurred during the third stage without a climacteric. There is no conversion of insoluble carbohydrates, such as starches into di- or monosaccarides, and the changes of insoluble protopectins to the soluble forms are not clear cut (18).

In general, the stage of maturity, or ripeness, of citrus fruits is determined on the ratio of total soluble solids to acids. The concentration of the soluble solids and sugars increases and the acids decrease in the pulp during growth and maturity of the fruit (42, 54, 94). This increase is apparently at the expense of the organic acids synthesis and changes in cell wall constituents (96). The reducing sugars comprise approximately 50% of the total sugar with the ratio remaining fairly constant during the later stages of development. The concentration of reducing sugars is more variable than that of the non-reducing, probably due to the fact that this carbohydrate fraction is readily metabolized (94).

Maximum amounts of free acids in 'Valencia' orange fruit were found to develop early in the season and to change very little up to maturity (83). Varma and Ramakrishnan (101) found that in citrus fruits having a diameter of 1.5 cm or greater citric acid was the predominant acid with small amounts of malic acid present. During stage I of fruit growth, succinic acid was the predominant one. Total acidity reached its peak in concentration in early fall and then was respired and/or diluted by an increase in vacuolar water as the juice vesicles increased in size and became highly vacuolated. During ripening, a further decrease in total acidity was observed (42).

The mechanism(s) responsible for acid accumulation in citrus fruits have been examined by Buslig (23). Generally, it is believed that organic acid accumulation is caused by overproduction of a specific acid which cannot be efficiently metabolized. Buslig found that citrate is metabolized by sour varieties less efficiently than by the not-quite-so-acid fruits. This was due in part to an altered control of the enzyme citrate synthetase in the more acid fruits. The energetics of acid accumulation may be accounted for by the high levels of ATP and the high ATP/ADP ratios in sour fruits during the greatest increase in citric acid content. ATP is believed to be required for the transport of acids from the mitochondria to the vacuole for subsequent accumulation.

Water Uptake

Fruit enlargement is accompanied by a large uptake of water. Up to 90% of the fresh weight can be attributed to water (75). Bain (6) reported that the enormous increase in fresh weight during stage II was primarily due to an accumulation of water in the pulp

segments. The water content can fluctuate significantly during growth and development, especially during a period of drought. Barthalomew and Reed (8), working with lemons, found that leaves may withdraw considerable amounts of water from the fruit when water cannot be supplied by the roots.

Role of Nucleic Acids in Growth and Development

Nucleic acids and their derivatives are constituents of every living cell, and probably constitute one of the most important groups of compounds affecting growth and development. These compounds direct the synthesis of protein and in many instances control various metabolic reactions. The suggestion has been made that the action of growth hormones may be mediated through an affect on nucleic acid metabolism (57).

As outlined in several biochemical texts (16, 29, 72), the following relationships seem to be well established. Two types of nucleic acids are known to occur in the living cell. One, DNA, is a chief constituent of the cell nucleus, and is concerned with the storage and transfer of genetic information. The other, RNA, is contained primarily in the cytoplasm outside the nucleus, and is responsible for directing the synthesis of both catalytic (enzymes) and structural proteins.

Both RNA and DNA are polymers of high molecular weight. The hydrolysis of these nucleic acids yields phosphorylated nucleotides, which consist of a ribose (RNA) or a deoxyribose (DNA) sugar, a phosphate moiety, and a nitrogenous base of either a purine or pyrimidine. The mononucleotides of RNA occur freely in the cytoplasm or combined with other constituents to form coenzymes essential for various bio-

chemical reactions. The physiological significance of these nucleotides and coenzymes is undoubtedly great since they are essential parts of many transfer reactions.

The synthesis and degradation of cellular constituents occur simultaneously in living cells. In some cases, the energy released from degradation reactions may be utilized in the synthesis of other cellular constituents, thereby maintaining an 'energy cycle'. Compounds which repeatedly function in linking endogenic reactions to exogenic reactions are the phosphorylated nucleosides and the pyridine nucleotides. Several biological processes in which these compounds are of importance are: photosynthesis, respiration, and metabolism of proteins, carbohydrates, and lipids.

The pyridine nucleotides, NAD and NADP and their reduced forms, are ubiquitous in the living cell. These compounds are coenzymes for the dehydrogenase enzymes which catalyze oxidation-reduction reactions. In the organic acid cycle, four of the five enzymes involved in the oxidation of pyruvic acid utilize NAD as an oxidizing agent. The pentose phosphate pathway is an important source of the reduced form of NADP (NADPH), which is required for the synthesis of fatty acids and some amino acids. In both the glycolytic cycle and the photosynthetic carbon reduction cycle, the reduced and oxidized forms of NAD and NADP are utilized.

The phosphorylation reactions are responsible for the production of energy rich compounds, ATP, GTP, etc. This process also utilizes both phosphorylated nucleotides and pyridine nucleotides. The pyridine nucleotides are important intermediates in the electron transport system of oxidative phosphorylation. These compounds are essential for the metabolism of carbohydrates, proteins, lipids, and nucleic acids.

Synchronization of the numerous biological reactions requires control mechanisms. In many reactions, various phosphorylated nucleotides and pyridine nucleotides could serve this specific function. In glycolysis the rate of the reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and pyruvic kinase is determined by the relative amount of ADP present. The activity of two enzymes, glycogen phosphorylase and phosphofructokinase, is regulated by AMP. AMP also serves as a regulator of the enzyme isocitrate dehydrogenase in the citric acid cycle. ATP is a negative effector of the enzyme phosphofructokinase (17, 29).

Probably, the most well defined relation between plant growth and development and nucleic acid metabolism is in the synthesis of protein. Both cell division and cell elongation are accompanied by a synthesis of both structural and catalytic protein (53, 56, 85). The current concept is that protein synthesis requires 3 distinct kinds of polymeric RNA; rRNA, sRNA and mRNA. Phosphorylated nucleotides are also essential in the energy transfer reactions. The different species of RNA required have been isolated from plant tissue and identified with the use of gel electrophoresis (47, 49, 68, 69, 103). Electrophoretic analyse show rRNA and sRNA. The third RNA component essential for protein synthesis, mRNA, is not readily distinguishable on electrophoretograms with UV analyses because of the amount present in the cell. However, it is distinguishable with radioactive labeling. Two types of rRNA are found in the green plant tissue, cytoplasmic rRNA and chloroplastic rRNA. The latter is found primarily in photosynthetic tissue. The RNA of each of these ribosomal units appears as a distinct fraction having a specific molecular weight. The rRNA of the cytoplasm is

comprised of a 25S and 18S RNA subunit and the chloroplastic rRNA comprised of a 23S and 16S RNA subunit. The 2 RNA subunits combine with protein to form the ribosome (3).

The synthesis of protein involves several steps. The initial step of protein synthesis involves mRNA. The genetic information residing in the DNA molecule is transferred to the mRNA. The mRNA attaches to the ribosome and directs the synthesis of protein. The transfer of the amino acids to the protein synthesizing site is accomplished via attachment of a specific tRNA. The attachment and subsequent release of the amino acids require both enzymes and high energy nucleotides (26).

In recent years, numerous papers and reviews have been compiled on the relation of nucleic acid metabolism to the action of plant hormones (34, 57, 58, 59, 74, 88). Furthermore, many workers have interpreted these results as indicating a dependency on nucleic acid metabolism for the manifestation of biological response to plant hormones. Hormones appear to affect the kind and amount of nucleic acids being synthesized by the plant (57).

Silberger and Skoog (93) in 1953, first demonstrated that auxin affects nucleic acid metabolism. Since then, the importance of RNA metabolism in auxin action has been confirmed by a number of workers (40, 60, 61, 108). Key and Shannon (61) observed that IAA at concentrations that promoted cell elongation in excised soybean hypocotyl tissue enhanced the incorporation of C^{14}-nucleotides into RNA. IAA- induced RNA synthesis has been reported to be required for cellulase synthesis and lateral cell expansion (32). There are several reports on the characterization of the newly synthesized RNA induced by auxin. These

studies indicate that auxin brings about the synthesis of all the major classes of RNA including DNA-RNA complex (59). Evidence has been accumulated by several workers which indicates that plant tissues are capable of incorporating cytokinins into RNA (35, 36). The occurrence of cytokinins in certain species of tRNA suggests that cytokinin action could possibly be connected to translational processes of protein synthesis. However, there is no evidence that cytokinin activity in growth regulation is associated with its presence in tRNA (57). Investigations on the sites of action of the gibberellins have shown that gibberellins affect almost all plant tissues and organs (81). Available information does indicate a possible interrelationship with nucleic acid metabolism. Like other growth regulators gibberellin has been found to enhance the synthesis of RNA (24) and the incorporation of labeled precursors into RNA (25, 33, 34). These effects are primarily associated with the retardation of senescence (10, 33, 34, 50) and the synthesis of α-amylase (80, 102).

Several studies have shown that the level of RNA could possibly be used as an indicator of the metabolic condition of cells at various stages of development and senescence (50, 73, 95, 99, 103). In general, a transition from an active to a more quiescent metabolic state is reflected in a decline in RNA synthesis. The most drastic changes in RNA are seen in rapidly senescing tissue. Changes associated with senescence can be controlled to some extent with the use of various growth regulators, e.g., GA and cytokinins. The action of these compounds is thought to be via maintenance of the functional state of RNA (33, 34, 50, 79). Ku and Romani (63) reported that the onset of senescence in the pear fruit is associated with a rapidly decreasing

rate of ribosomal turnover which leads to a complete cessation of rRNA and ribosomal synthesis as fruits reach the climacteric. Richmond and Biale (84) have suggested that the critical intercellular transitions occurring during the latter phase of the climacteric may stem from a reduction of RNA synthesis.

The objective of this study was to investigate the quantitative and qualitative changes of RNA and acid-soluble nucleotides during stages II and III of citrus fruit growth and development. Changes in these constituents could serve as an index to the various biochemical and physiological processes associated with citrus fruit growth, maturation and senescence.

MATERIALS AND METHODS

Plant Materials

Citrus fruit varieties, listed in Table 1, were harvested from a commercial planting at Citra, Florida,and from a planting at the University of Florida, Gainesville. Fruit were collected periodically to encompass varying physiological ages from the early portion of stage II to well into stage III (6). Samples of each variety were collected to minimize locational differences by sampling in pre-selected quadrants of each tree each time. Samples were quick frozen with dry ice soon after harvesting. Fruit growth was measured as an increase in volume.

Total Soluble Solids and Titratable Acidity Measurement

Total soluble solids were determined using a Bausch and Lomb refractometer, model ABBE 3L, and acidity was determined by titration.

Preparation of Tissue Extracts

Frozen fruit were peeled, sectioned and ground with a mortar and pestle in dry ice. The powdery pulp was kept frozen until needed.

Determination of Total RNA Concentration

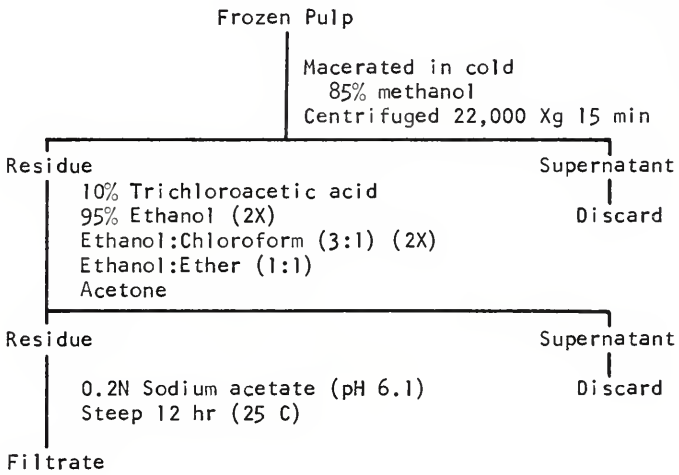
Numerous procedures have been used for the extraction of RNA from plant and animal tissue, but most are based on the use of acids (100), phenol and a suitable buffer (68), and neutral salts (76), or a combination of these extractants. In this study, the extraction procedure

Table 1. Experimental material

	Type	Description
1.	'Orlando' tangelo	*Citrus reticulata* x *C. paradisi*
2.	'Hamlin' orange	*C. sinensis* (L.) Osbeck
3.	'Pineapple' orange	*C. sinensis* (L.) Osbeck
4.	'Parson Brown' orange	*C. sinensis* (L.) Osbeck
5.	'Calamondin' orange	*C. madurensis* Lou.

of Holdgate and Goodwin (45) was used for quantitation of total RNA. The technique of Holdgate and Goodwin permitted reliable estimation of RNA in citrus fruit pulp without the use of elaborate and time consuming procedures.

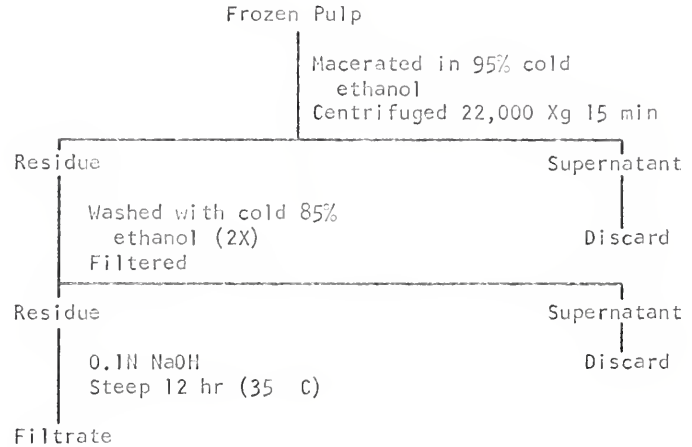
Frozen pulp tissue was combined with cold 85% methanol and macerated in a Sorvall stainless steel omni-mixer (5 min at 8,000 rpm), and extracted as follows:



The concentration of RNA was measured by scanning (220 nm to 320 nm) the RNA extract using a Beckman DB-G spectrophotometer. Yeast RNA (P-L Biochemical Laboratories) was used as a standard.

Determination of Protein Concentration and the Percent AISR

Frozen pulp was combined with cold 95% ethanol and macerated in a Sorvall stainless steel omni-mixer (5 min at 8,000 rpm) kept below 4 C. The subsequent extraction procedure was as follows:

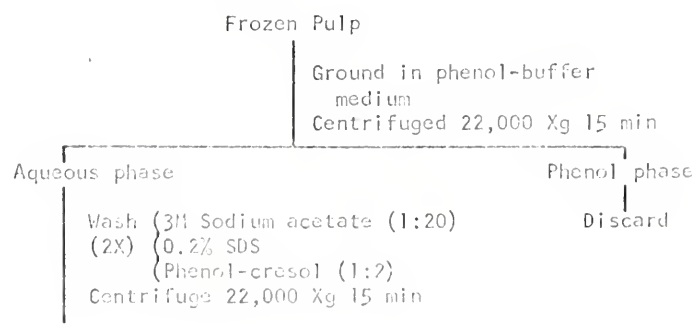


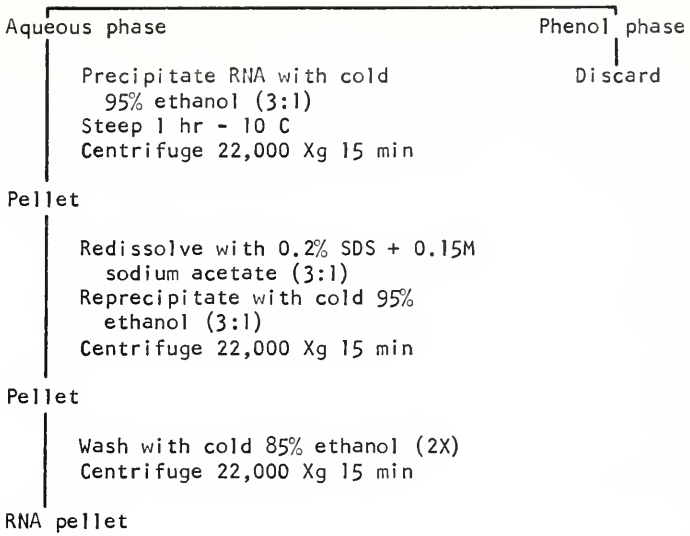
Protein concentration in the final filtrate was measured by a modified Lowery method described by Potty (82). Bovine serum albumen was used as the standard. The percent AISR was also determined from this fraction.

Isolation of Cytoplasmic Ribosomal and Soluble RNA

Preparation of RNA

A modified procedure described by Loening (68) was used. Frozen fruit pulp was ground in a phenol-buffer medium in a Sorvall stainless steel omni-mixer (5 min at 8,000 rpm) kept at 4 C, and the subsequent extraction procedure was followed:




Aqueous phase
Phenol phase
Discard
Precipitate RNA with cold 95% ethanol (3:1)
Steep 1 hr - 10 C
Centrifuge 22,000 Xg 15 min
Pellet
Redissolve with 0.2% SDS + 0.15M sodium acetate (3:1)
Reprecipitate with cold 95% ethanol (3:1)
Centrifuge 22,000 Xg 15 min
Pellet
Wash with cold 85% ethanol (2X)
Centrifuge 22,000 Xg 15 min
RNA pellet

The RNA pellet was redissolved in 2.5 ml of the electrophoresis buffer containing 6% sucrose (RNAase free) and 0.2% SDS. This suspension was used for electrophoresis.

Preparation of gels

Gels (7.5 x 0.6 cm) of 2.6% polyacrylamide were prepared according to Loening (68). Gel materials were obtained from Bio-Rad Chemical Corporation. Gels were stored in the electrophoresis buffer overnight before using.

Procedure for electrophoresis

The buffer for electrophoresis was prepared according to Loening (68). Electrophoresis was carried out at room temperature. Current (5 mAmps/gel) was applied for 1 hr to the gel to remove the polymerization catalyst and other impurities. The RNA suspension (50 ul) was layered on the gel and electrophoresis was continued. Suitable separation of the rRNA subunits and sRNA was obtained in 70 min.

Scanning of ultraviolet absorption

UV absorption zones on gels were determined using a Beckman DU spectrophotometer equipped with a Gilford Gel Scanner (Model 2000). Gels were scanned at 260 nm at a rate of 2 cm/min with a slit width of 0.35 mm.

Incorporation of ^{32}P into RNA

Calamondin fruit, 1.5 and 3.0 cm in diameter at completion of fruit growth and before color break, and mature fruit prior to abscission, were labelled with ^{32}P orthophosphate applied to the stem end for 24 hr. The fruits were quick frozen with dry ice and the RNA extracted as previously described.

Radioactivity determination

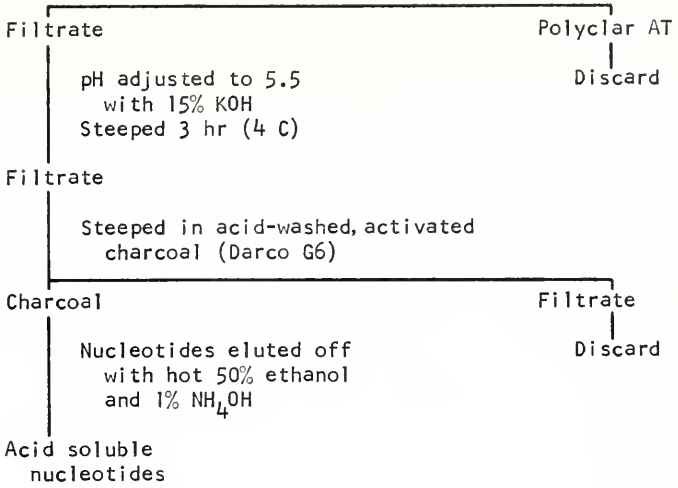
Gels were frozen on a glass plate with dry ice. The ^{32}P-labelled gels were cut into 2 mm sections using a micrometer equipped with a guillotine cutting apparatus. The sections were digested in 0.5 ml of 30% hydrogen peroxide at 70 C overnight in scintillation vials. Counting was done in 12 ml Aquasol (New England Nuclear) using a refrigerated Packard Tri-Carb scintillation counter, model 314 EX.

Determination of Acid-Soluble Nucleotides

Frozen pulp was combined with cold 0.6 N perchloric acid and ground in a Sorvall stainless steel omni-mixer at 4 C. The macerate was steeped for 3 hr at 4 C and the following extraction procedure was followed:

Frozen Pulp

| 0.6 N Perchloric acid (3:1)
| Filtered through bed of Polyclar
| AT (GAF Corporation) (2X)



Citrus pulp contains large quantities of polyphenols, and removal of these compounds with Polyclar AT was required for maximum resolution with UV analysis. Removal of the acid-soluble nucleotides by Polyclar AT, a weak anion-exchange resin, was insignificant as determined using radioactive standards to check the procedure.

The final extract was reduced to 10 ml and placed onto a Dowex 1X8 (200-400 mesh) formate ion-exchange column (0.9 cm x 13 cm). Chromatography was begun with a 2-step elution system using 2 chambers to produce a curvilinear gradient. For range 1, the mixing chamber initially contained 250 ml of distilled water and the reservoir 250 ml of 4 N formic acid. For range 2, the mixing chamber contained 250 ml of 4 N formic acid and the reservoir 250 ml of 1.6 N ammonium formate. The eluate from the column was continuously monitored at 260 nm using a Gilford analyzer (Model 2000) fitted to a Beckman DU spectrophotometer. Any material not adsorbed onto the column was eliminated by washing with distilled water prior to beginning the gradient. The 5' mononucleotides (P-L Biochemical Laboratories) were used as standards.

Tentative identification was made by comparing the relative position of elution from the column with standards fractionated by the same procedure. Further identification was made by UV absorption spectral analysis and thin-layer chromatography where possible. Thin-layer chromatographic analysis was made using avicel plates prepared in the laboratory and developed in a solvent system consisting of isobutyric acid, conc. ammonium hydroxide, and water (57:4:39 v/v). Qualitative determination was made by UV visualization.

Determination of ATP Concentration

Concentration of ATP in the fruit pulp was measured by a modified firefly luciferin-luciferase system described by Buslig and Attaway (24). Each batch of firefly extract was standardized with known amounts of ATP.

RESULTS

Changes in soluble solids, titratable acidity, and fruit volume were determined as reference points for the work on nucleotides and nucleic acids.

Fruit Growth

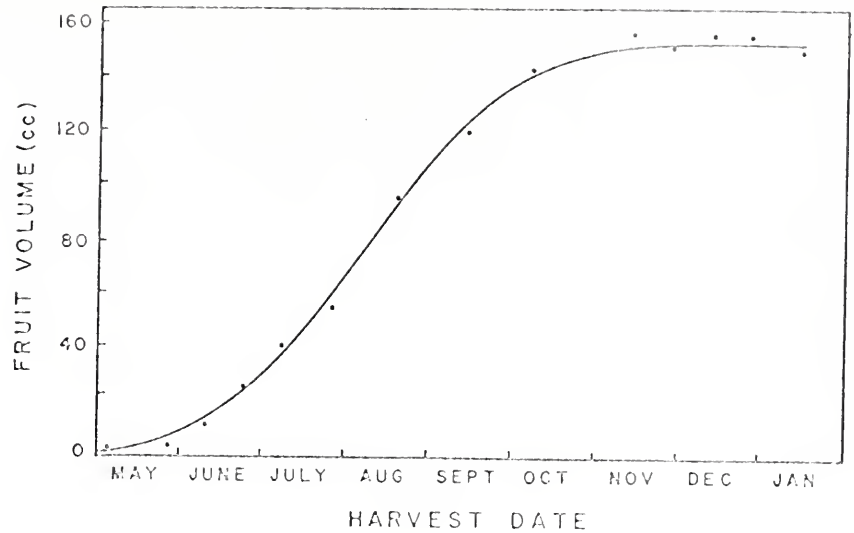
The flowering period of the 4 varieties studied was between March 9 and March 30, 1970. The growth curves of these varieties are presented in Figs. 1, 2, 3, and 4. Fruit size increased until early November for 'Orlando' tangelo, mid-November for 'Hamlin' and 'Pineapple', and late November for 'Parson Brown' oranges. The stages of fruit growth of citrus have been studied by Bain (6). This study was concerned with changes occurring during stage II, cell enlargement, and into stage III, fruit maturation.

Percent Soluble Solids and Titratable Acidity in Citrus Fruit Pulp at Various Stages of Growth and Development

Sugar accumulation increases with growth. The increase in sugars has been suggested to take place at the expense of cell wall constituents and decreased acid synthesis (96). The percent soluble solids and titratable acidity during fruit growth and development of each variety studied are presented in Tables 2 and 3, respectively. The major constituents of the soluble solids are the sugars, primarily sucrose, glucose and fructose (94). As shown in Table 2, the percent soluble solids increased during the latter part of stage II and early

Fig. 1. Growth curve of 'Orlando' tangelo fruit.

Fig. 2. Growth curve of 'Hamlin' orange fruit.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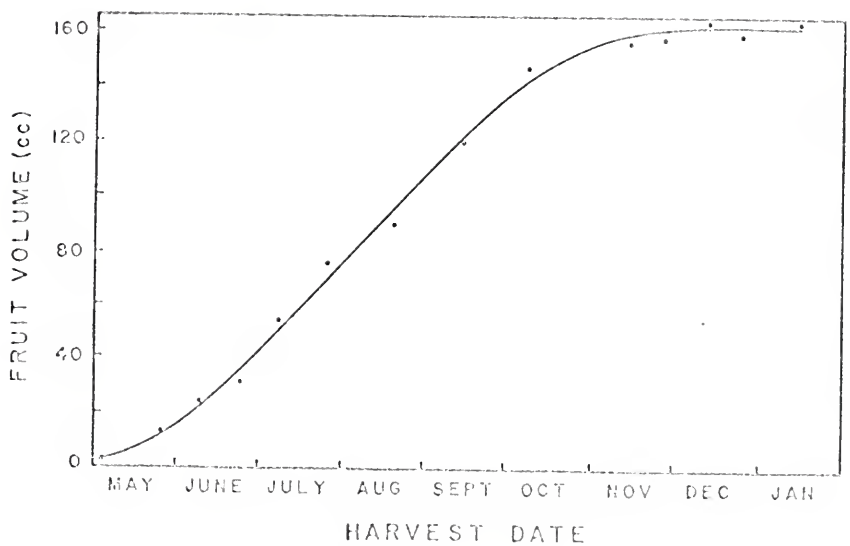
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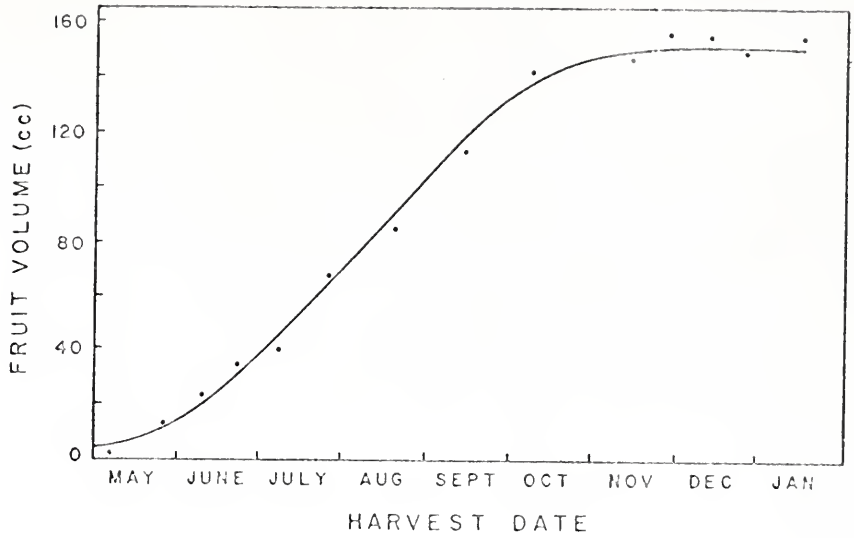
Fig. 3. Growth curve of 'Pineapple' orange fruit.

Fig. 4. Growth curve of 'Parson Brown' orange fruit.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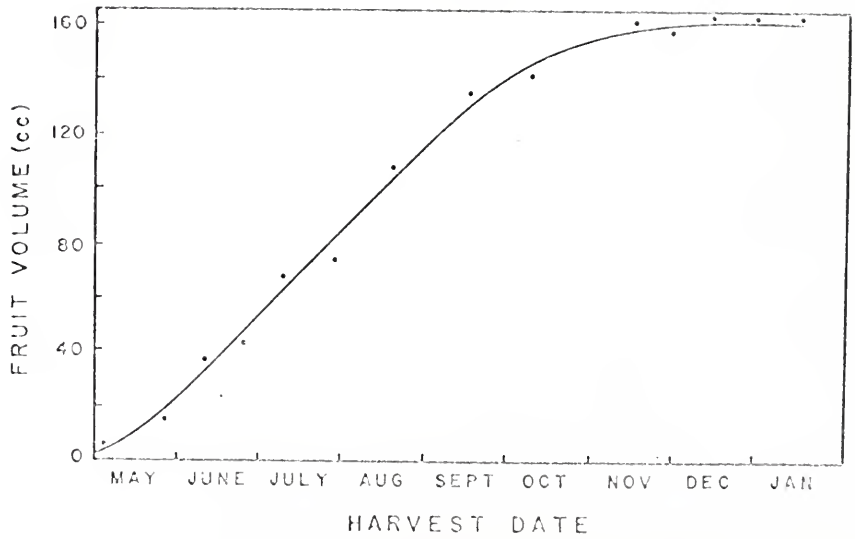
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Table 2. Soluble solids in citrus fruit pulp at various stages of growth and development

Harvest Date	% Soluble Solids			
	'Orlando' tangelo	'Hamlin' orange	'Pineapple' orange	'Parson Brown' orange
9-26-70	6.70	---	---	---
10-10-70	9.20	12.30	10.30	9.80
11-14-70	11.29	12.56	11.10	13.20
11-25-70	11.40	13.20	13.60	14.60
12-12-70	10.30	12.00	11.30	12.00
12-22-70	---	13.00	11.70	12.10
1-17-71	---	---	11.80	12.30

Table 3. Titratable acidity in citrus fruit pulp at various stages of growth and development

Harvest Date	% Titratable Acidity	Harvest Date	% Titratable Acidity
'Orlando' tangelo		'Hamlin' orange	
7-23-70	1.37	7-23-70	2.00
8-19-70	1.26	8-19-70	1.90
9-26-70	0.96	9-11-70	1.18
10-10-70	0.94	10-10-70	0.93
11-14-70	0.87	11-14-70	0.92
11-25-70	0.90	11-25-70	0.96
12-12-70	0.92	12-12-70	1.13
		12-22-70	0.78
'Pineapple' orange		'Parson Brown' orange	
7-08-70	1.35	7-08-70	2.09
7-23-70	1.34	7-23-70	2.02
8-19-70	1.22	8-19-70	2.00
9-26-70	1.22	9-26-70	1.52
10-10-70	0.95	10-10-70	1.34
11-14-70	1.01	11-14-70	1.16
11-25-70	1.02	11-25-70	1.17
12-22-70	0.94	12-22-70	1.01
1-17-71	0.89	1-17-71	1.07

part of stage III of fruit growth. These results are in agreement with those reported by others (9, 42). The relatively high soluble solids level occurring on November 25, 1970, for all varieties is attributed to an extended warm period preceding this date.

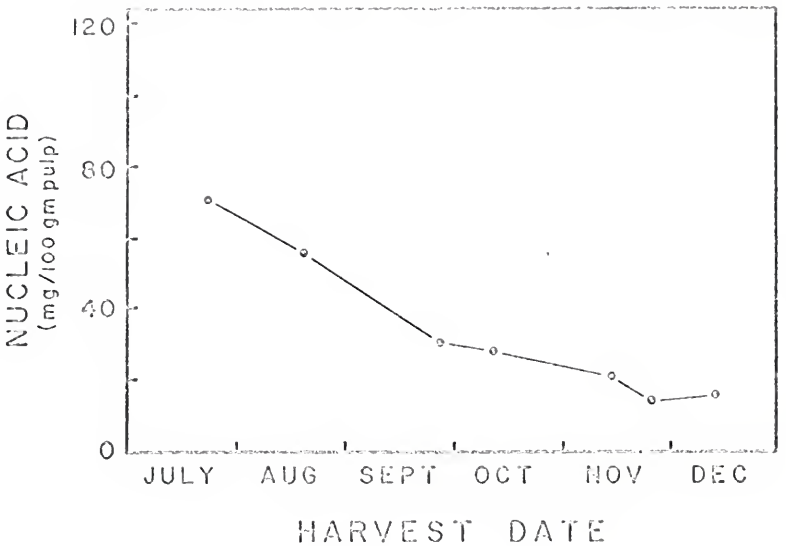
The acidity in citrus fruit pulp is due principally to citric acid with small amounts of malic, tartaric and succinic acid also present (15). Citric acid concentration was greatest in young developing fruits, decreasing with fruit growth (Table 3). The decrease in acidity was more rapid in the early part of stage II, July 8, 1970, to October 10, 1970.

RNA Concentration in Citrus Fruit Pulp at Various Stages of Growth and Development

Since RNA is required for protein synthesis, determinations were made of the RNA fraction. The relative concentration in mg/100 g pulp in the fruit pulp of the 4 varieties during growth and development are shown in Figs. 5, 6, 7, and 8. The RNA concentration in all varieties decreased with growth and development during the period studied. The greatest change in concentration occurred in the fruits of 'Orlando' tangelo and 'Parson Brown' oranges. The RNA concentration range from young fruit to mature fruits was 72 to 14 mg for 'Orlando' tangelo and 101 to 10 mg for 'Parson Brown' orange, respectively. The concentration range for 'Hamlin' orange fruit pulp was 82 mg in young fruit to 42 mg in mature fruit, and for 'Pineapple' orange 80 mg in young fruit to 20 mg in mature fruit. The concentration of RNA of 'Hamlin', 'Pineapple', and 'Parson Brown' orange fruits fluctuated appreciably during the period studied in contrast to that observed in fruits of 'Orlando' tangelo. Trees of the former 3 varieties were subjected to significant

Fig. 5. Total RNA concentration in the fruit pulp of 'Orlando' tangelo during growth and development.

Fig. 6. Total RNA concentration in the fruit pulp of 'Hamlin' orange during growth and development.


NUCLEIC ACID
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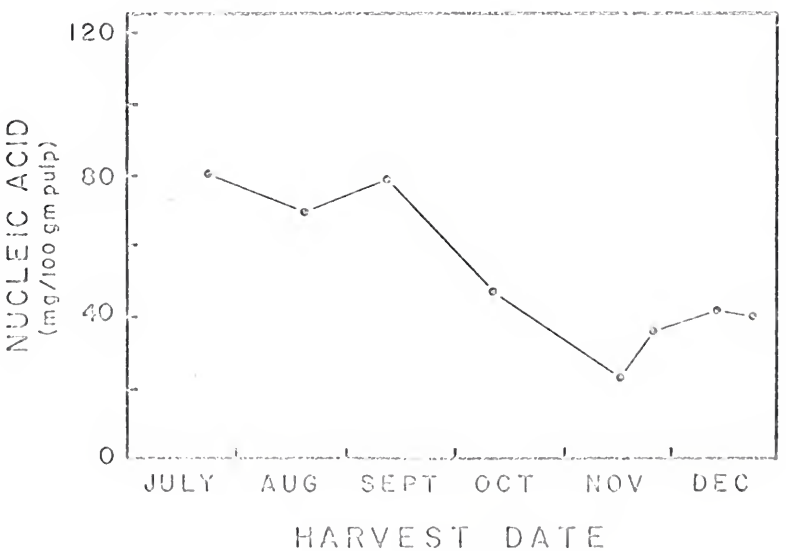
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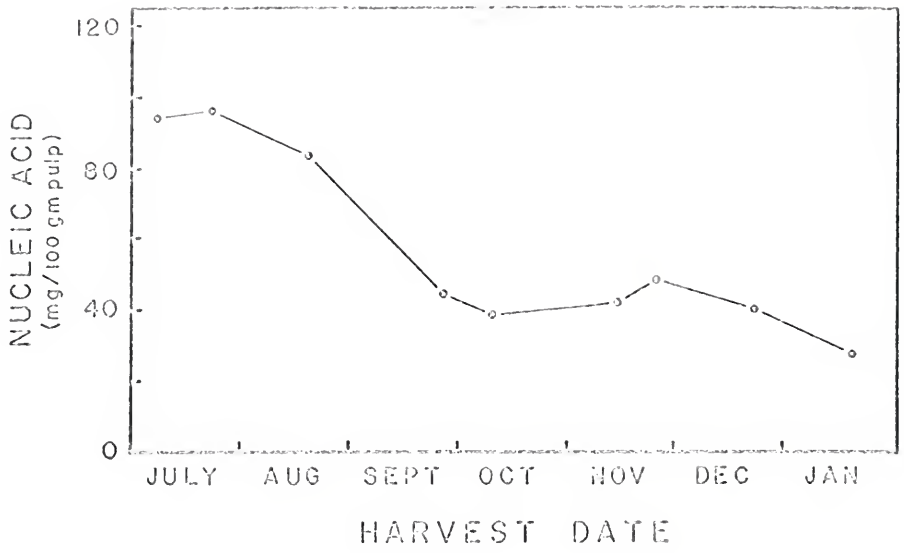
Fig. 7. Total RNA concentration in the fruit pulp of 'Pineapple' orange during growth and development.

Fig. 8. Total RNA concentration in the fruit pulp of 'Parson Brown' orange during growth and development.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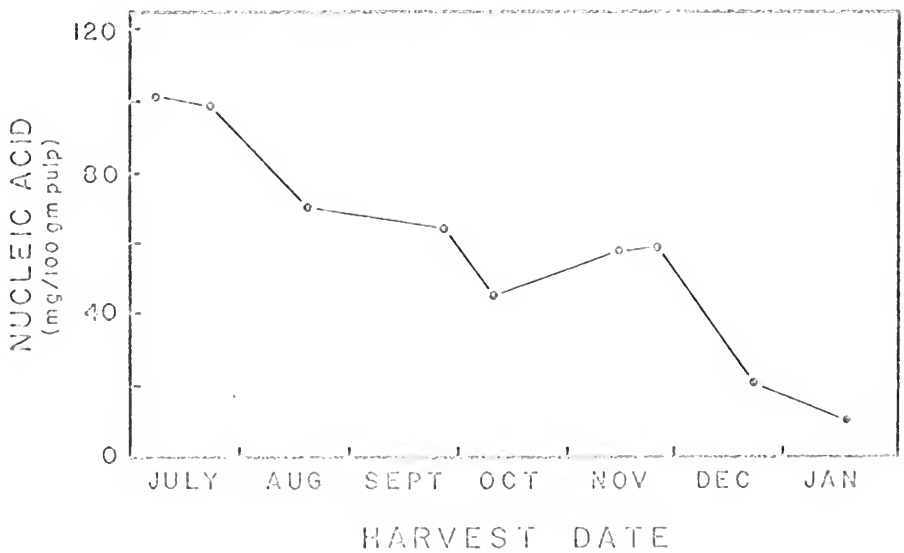
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fluctuations in the soil moisture during this period. Such conditions generally resulted in variations in the water content of the fruit. Barthalomew and Reed (8) have shown that fruits serve as a source of water for leaves under drought conditions. This would alter the concentration of the various cellular constituents.

In view of the fact that the water content varies with fruit growth, the RNA concentration based on the alcohol insoluble residue fraction (AISR) was also determined. All RNA is precipitated, along with other cellular constituents, by ethanol. These results are presented in Table 4. Fruits of 'Orlando' tangelo showed a gradual decrease in the RNA concentration with fruit growth and development ranging from 23.7 mg in young fruits to 11.2 mg/g AISR in mature fruits. The RNA concentration in 'Hamlin' orange fruits was constant between the harvest dates of July 23, 1970, to October 10 , 1970, at approximately 19.4 mg and decreasing to 13.2 mg prior to completion of fruit growth, at which time an increase occurred. The maximum concentration at maturity for the period studied was 24.3 mg. The concentration of RNA at the various stages of growth was similar for both 'Pineapple' and 'Parson Brown' fruits. The RNA concentration was constant prior to an increase that occurred at the early part of stage III. The average concentration prior to the increase was 21.1 mg/g of pulp for 'Pineapple' orange and 20.3 mg/g of pulp for 'Parson Brown' orange. At maximum accumulation, the RNA concentration was greater in fruits of 'Parson Brown' reaching a level of 48.5 mg in comparison to 26.6 mg in fruits of 'Pineapple' orange. Following this increase, the RNA concentration decreased in both varieties to 22.9 mg in 'Pineapple' and 7.9 mg in 'Parson Brown' oranges on the last date sampled, January 17, 1971.

Table 4. Concentration of RNA and protein in the alcohol insoluble residue (AISR) fraction of the citrus fruit pulp at various stages of growth and development

Harvest Date	AISR %	RNA mg/g	Protein mg/g	Harvest Date	AISR %	RNA mg/g	Protein mg/g
	'Orlando' tangelo				'Hamlin' orange		
7-23-70	3.0	23.7	261.4	7-23-70	4.2	19.5	180.1
8-19-70	2.9	18.4	250.2	8-19-70	3.7	19.1	172.6
9-26-70	1.9	16.1	299.3	9-11-70	4.0	19.8	147.1
10-10-70	1.7	15.5	288.3	10-10-70	2.3	20.7	238.5
11-14-70	1.4	15.7	336.1	11-14-70	1.7	13.2	299.8
11-25-70	1.3	11.2	379.2	11-25-70	1.8	20.5	305.0
12-12-70	1.3	12.4	379.2	12-12-70	1.8	22.1	273.3
				12-22-70	1.7	24.3	294.9
	'Pineapple' orange				'Parson Brown' orange		
7-08-70	4.6	20.4	153.4	7-08-70	5.0	20.3	141.7
7-23-70	4.1	23.4	172.2	7-23-70	4.9	20.2	144.0
8-19-70	3.9	21.2	160.9	8-19-70	3.7	19.5	174.8
9-26-70	2.1	21.4	224.1	9-26-70	2.0	21.0	226.9
10-10-70	2.2	20.7	260.8	10-10-70	1.8	25.1	283.2
11-14-70	1.8	21.3	250.5	11-14-70	1.4	48.5	350.1
11-25-70	1.7	26.6	276.8	11-25-70	1.5	43.3	319.3
12-22-70	1.6	25.0	306.3	12-22-70	1.4	13.6	344.1
1-17-71	1.4	22.9	368.6	1-17-71	1.3	7.9	379.2

Protein Concentration in Citrus Fruit Pulp at Various Stages of Growth and Development

Protein synthesis has been shown to be essential for phases II and III of fruit growth and development. The protein concentration in the pulp was determined since protein synthesis is regulated by RNA. The protein concentration based on the fresh weight of the fruit pulp is shown in Table 5 for the 4 varieties. The protein concentration decreased during fruit growth reaching a constant level at completion of fruit growth in all 4 varieties. The concentrations in g/100 g of pulp were similar for both 'Orlando' tangelo and 'Hamlin' orange fruits ranging from 0.784 g and 0.756 g in young fruit to 0.492 g and 0.501 g in mature fruit, respectively. The concentration range in fruits of 'Pineapple' and 'Parson Brown' oranges were also similar ranging from 0.706 g and 0.706 g in young fruit to 0.501 g and 0.493 g in mature fruit, respectively.

The protein concentration in the AISR fraction of the 4 varieties is presented in Table 4. The protein concentration began increasing approximately 2 months prior to completion of fruit growth, reaching a constant level at this time. The increase in protein was not as great in fruits of 'Orlando' tangelo, ranging from 261 mg in young fruit to 379 mg/g AISR in mature fruit. In fruits of 'Hamlin', 'Pineapple', and 'Parson Brown' oranges the protein concentration ranged from a minimum of 180 mg, 153 mg and 142 mg in young fruit to a maximum of 295 mg, 368 mg and 379 mg/g of AISR in mature fruit, respectively.

Fractionational Patterns of Cytoplasmic RNA from Citrus Fruit Pulp at Various Stages of Growth and Development

Recently, it has been demonstrated that certain changes in RNA may

Table 5. Protein concentration in the citrus fruit pulp at various stages of growth and development

Harvest Date	Protein g/100 g	Harvest Date	Protein g/100 g
'Orlando' tangelo		'Hamlin' orange	
7-23-70	0.784	7-23-70	0.756
8-19-70	0.725	8-19-70	0.638
9-26-70	0.568	9-11-70	0.588
10-10-70	0.490	10-10-70	0.549
11-14-70	0.471	11-14-70	0.510
11-25-70	0.493	11-25-70	0.549
12-12-70	0.493	12-12-70	0.498
		12-22-70	0.501
'Pineapple' orange		'Parson Brown' orange	
7-08-70	0.706	7-08-70	0.706
7-23-70	0.686	7-23-70	0.706
8-19-70	0.627	8-19-70	0.647
9-26-70	0.471	9-26-70	0.454
10-10-70	0.569	10-10-70	0.509
11-14-70	0.451	11-14-70	0.490
11-25-70	0.471	11-25-70	0.479
12-22-70	0.490	12-22-70	0.482
1-17-71	0.501	1-17-71	0.493

be good indices for cellular states of synthesizing or non-synthesizing systems (70, 84, 103). Electrophoretograms of polymeric RNA indicate that growing and mature fruits have markedly different polymer patterns. The RNA patterns of the 4 varieties studied are shown in Figs. 9 thru 26. It is assumed that the cytoplasmic rRNA subunits, 25S (Rf 0.23) and 18S (Rf 0.35), and sRNA (Rf 0.76) are the RNA species being separated in the electrophoretograms. In young fruit harvested during July and September, 2 minor components were present immediately following the 2 cytoplasmic subunits corresponding to the 23S (Rf 0.28) and 18S (Rf 0.41) subunits of plastid rRNA. The identity of the 2 minor components preceding the 25S subunit are unknown. DNA is reported to occur in this region (69).

The electrophoretograms show that during fruit growth and development both the number of components and the quantity of each component decreased in all varieties. Similar results have been reported in other types of plant tissue (47, 66, 68, 73). The possibility that RNA could have been degraded during extraction is ruled out since low temperatures and SDS, an enzyme inhibitor, were used throughout the extraction procedure. It is of special interest that plastid rRNA subunits appear to be present in young fruit but absent from mature fruit. The presence of these subunits indicate that plastids are present in the juice vesicles during the early stage of growth. The quantitative change is also noteworthy, because such changes indicate degradation. The electrophoretograms indicate that the quantity of each component decreased with growth reaching a constant level at completion of stage II.

Good criteria of the metabolic state of RNA are the ratios of the major polymeric RNA fractions. Several studies indicate that the ratio

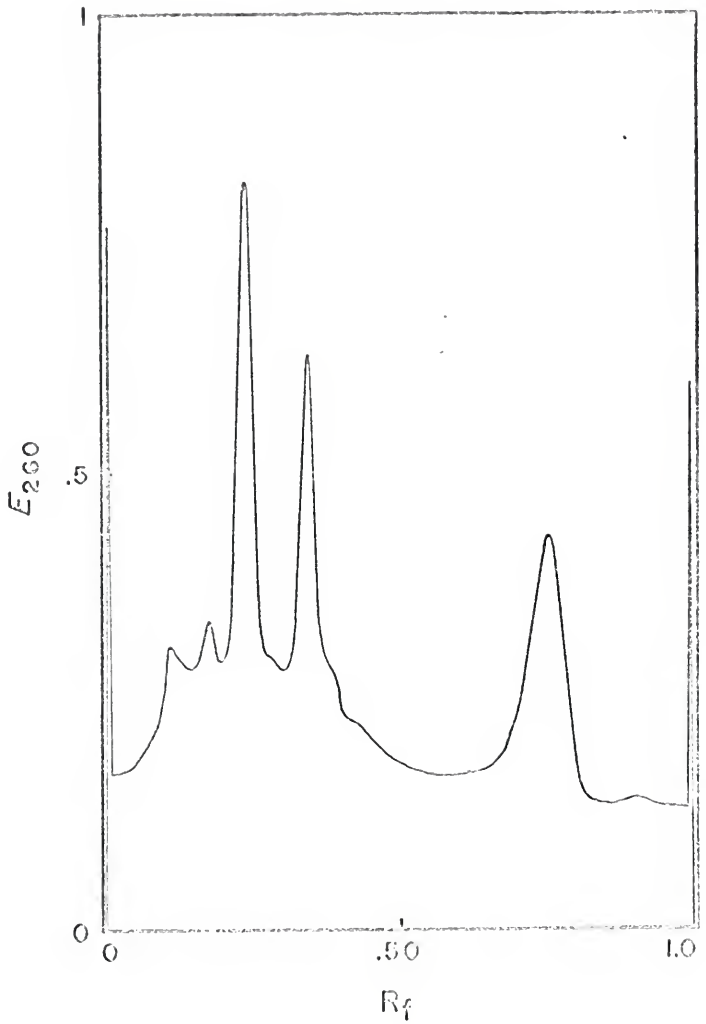


Fig. 9. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Orlando' tangelo fruits harvested 7-23-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

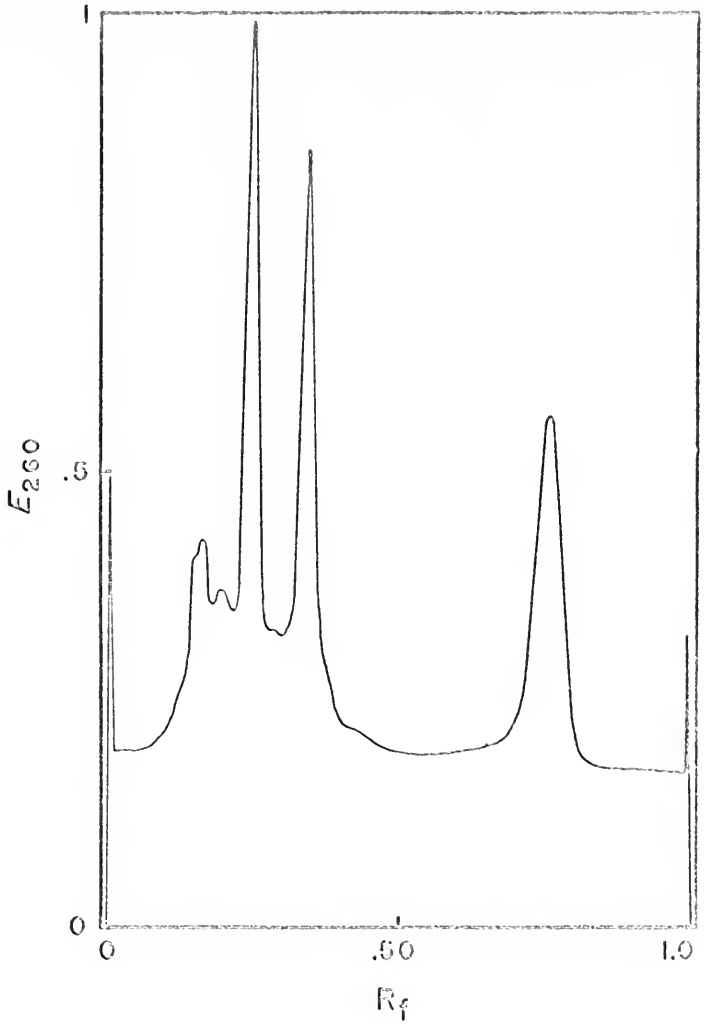


Fig. 10. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Orlando' tangelo fruits harvested 9-26-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

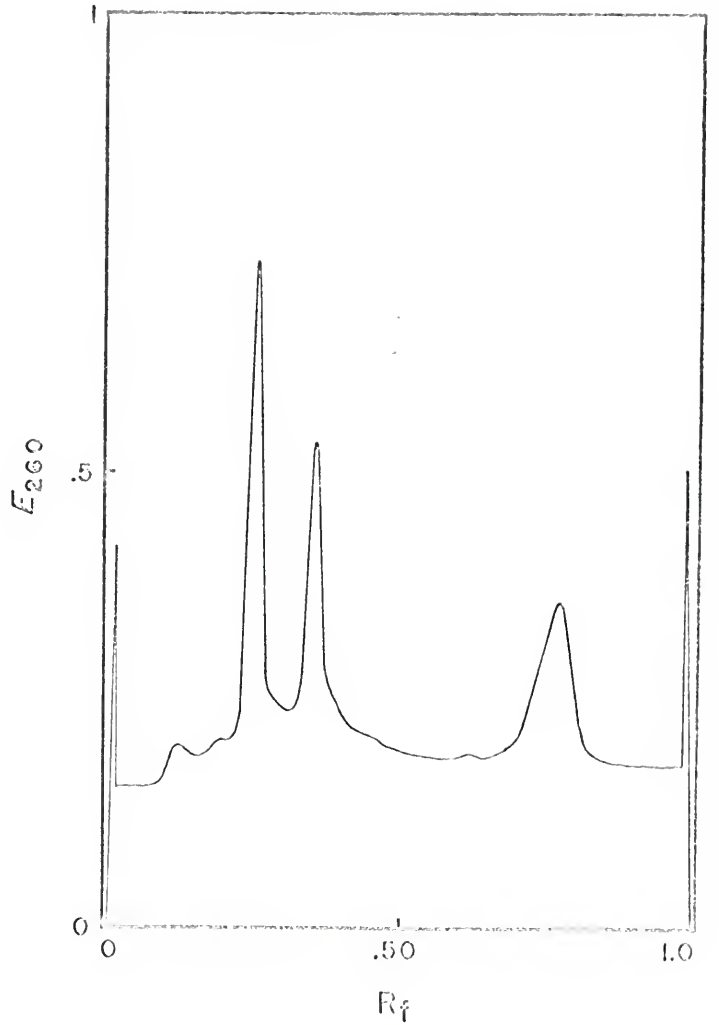


Fig. 11. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Orlando' tangelo fruits harvested 11-25-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

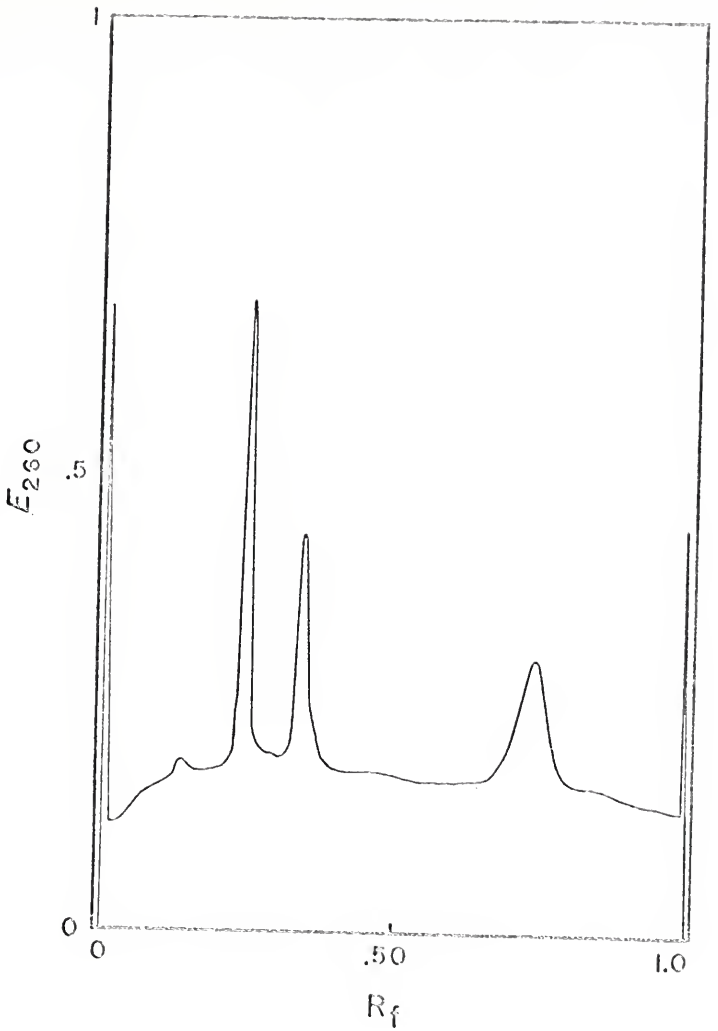


Fig. 12. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Orlando' tangelo fruits harvested 12-12-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

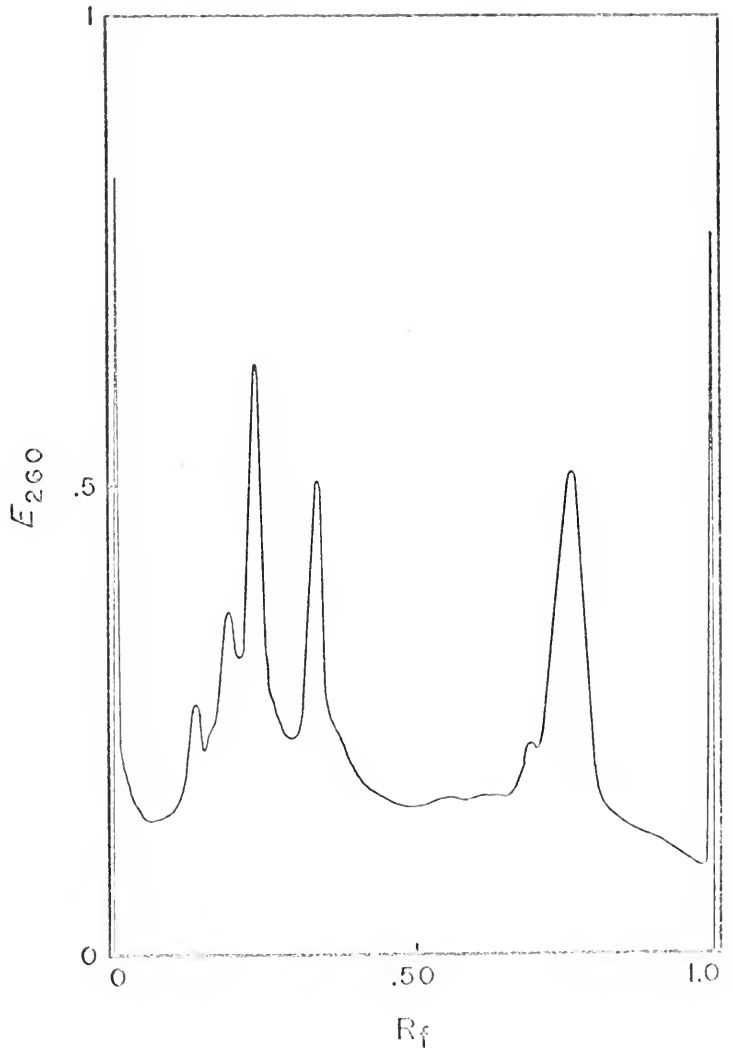


Fig. 13. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Hamlin' orange fruits harvested 7-23-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

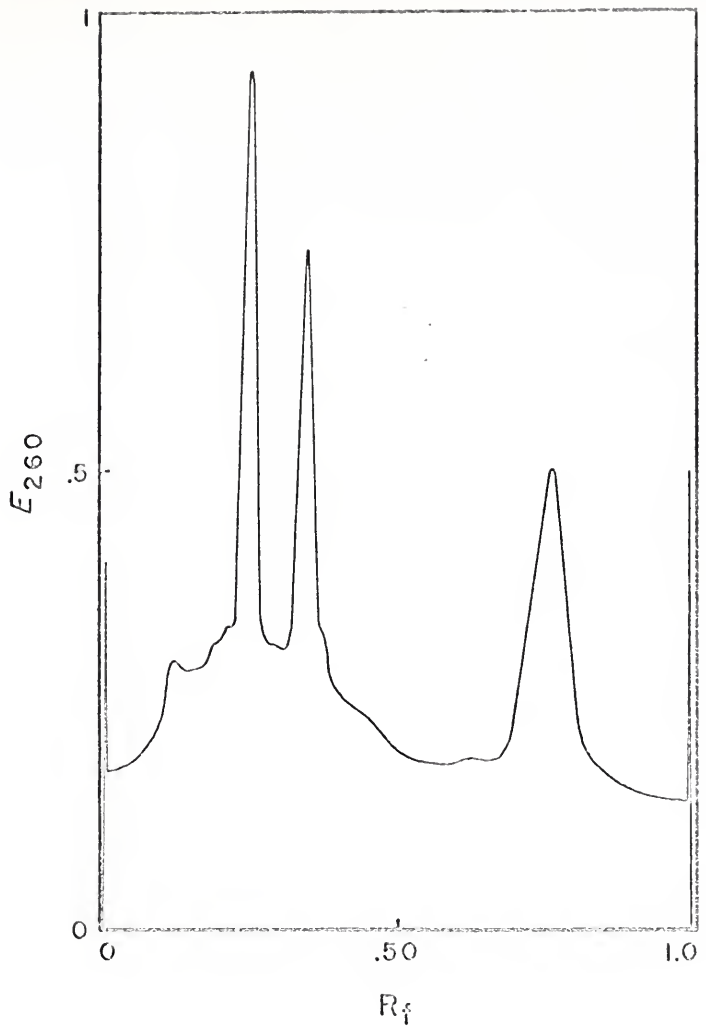


Fig. 14. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Hamlin' orange fruits harvested 9-11-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

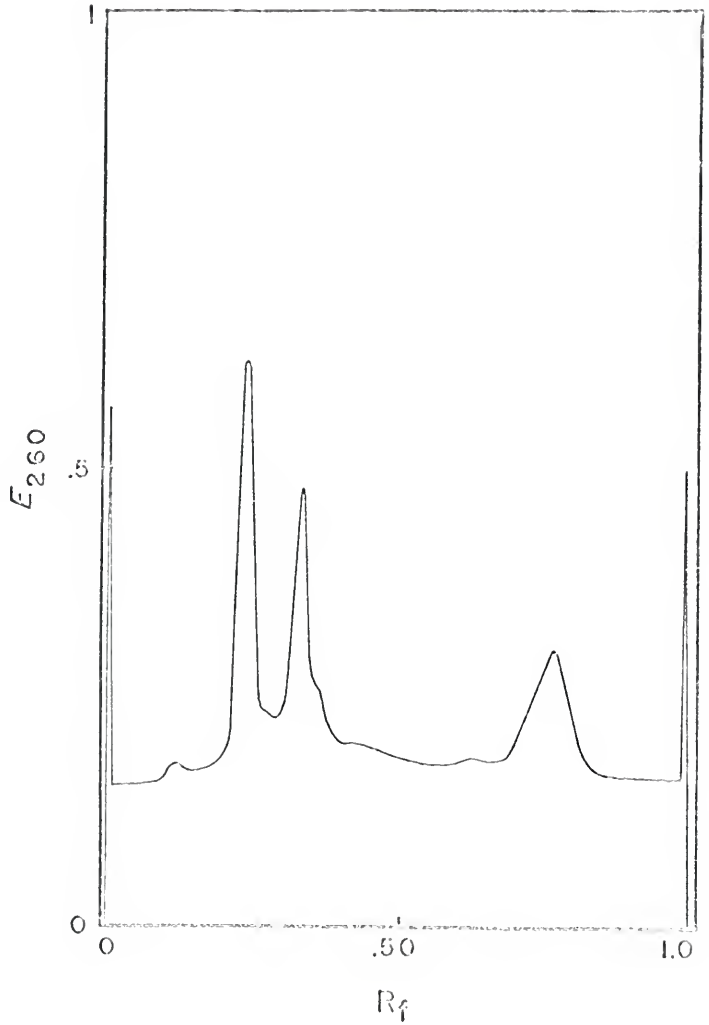


Fig. 15. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Hamlin' orange fruits harvested 11-25-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

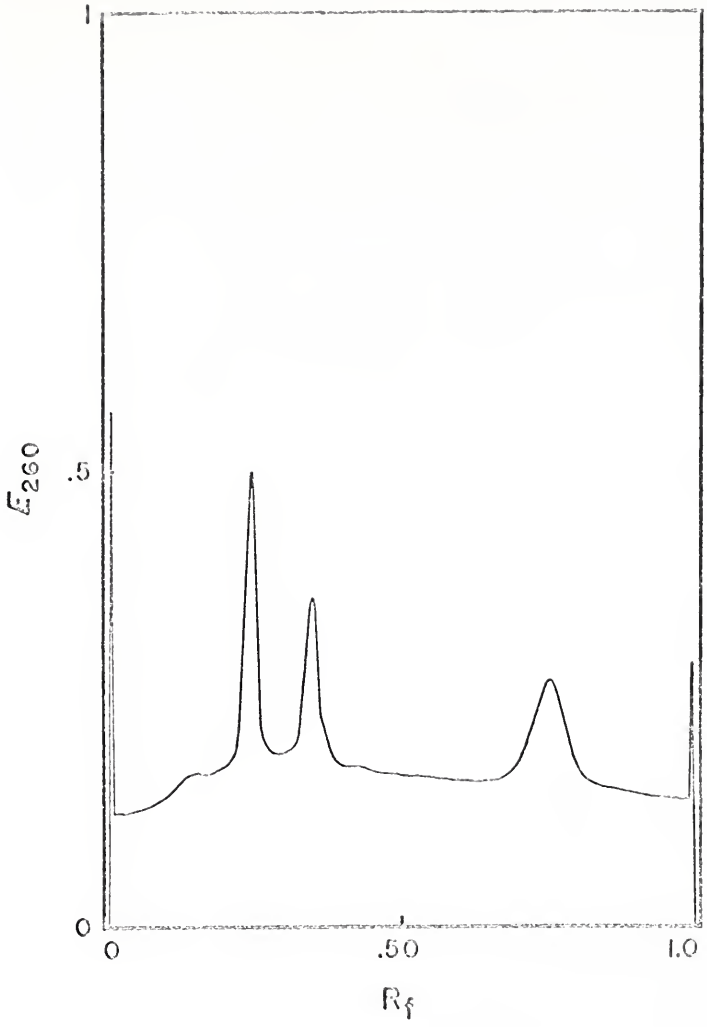


Fig. 16. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Hamlin' orange fruits harvested 12-22-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

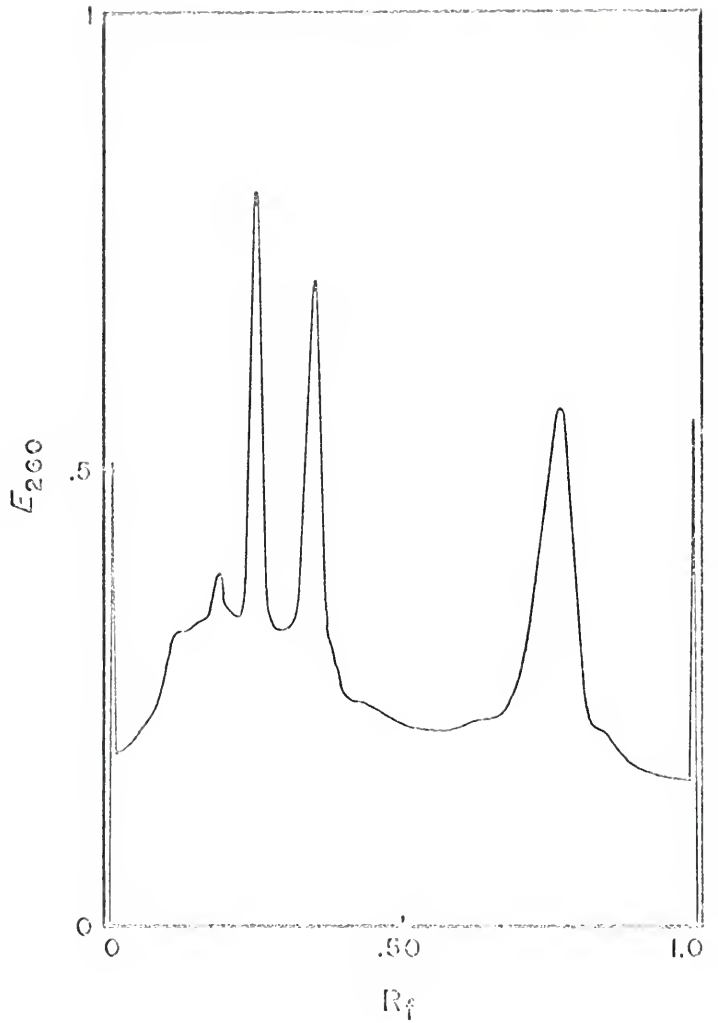


Fig. 17. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Pineapple' orange fruits harvested 7-23-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

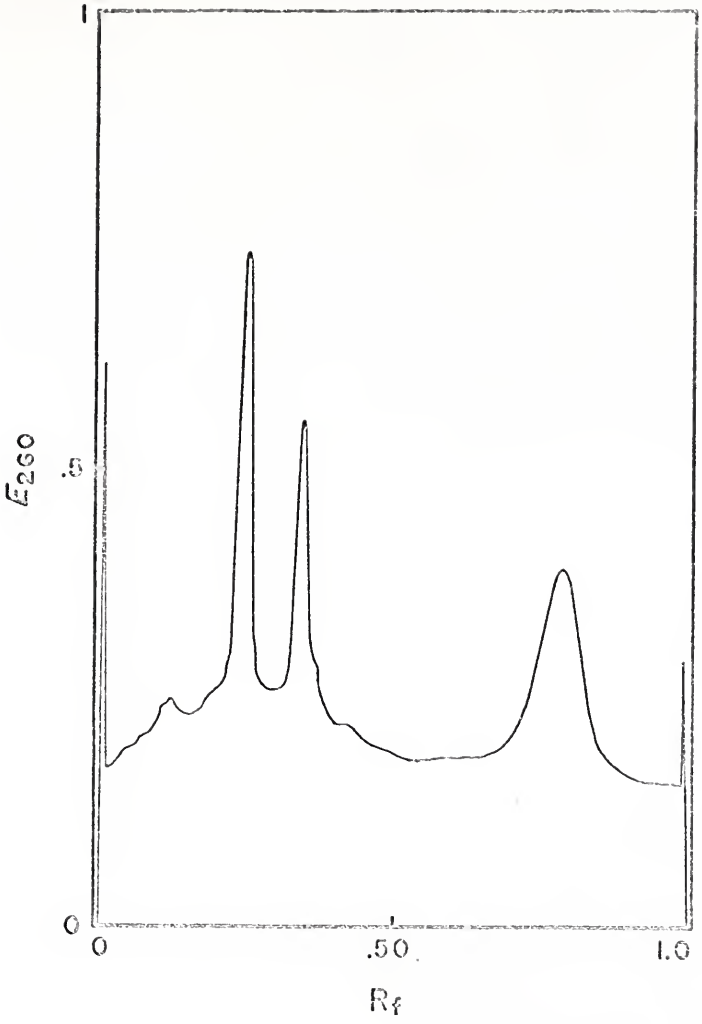


Fig. 18. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Pineapple' orange fruits harvested 9-26-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

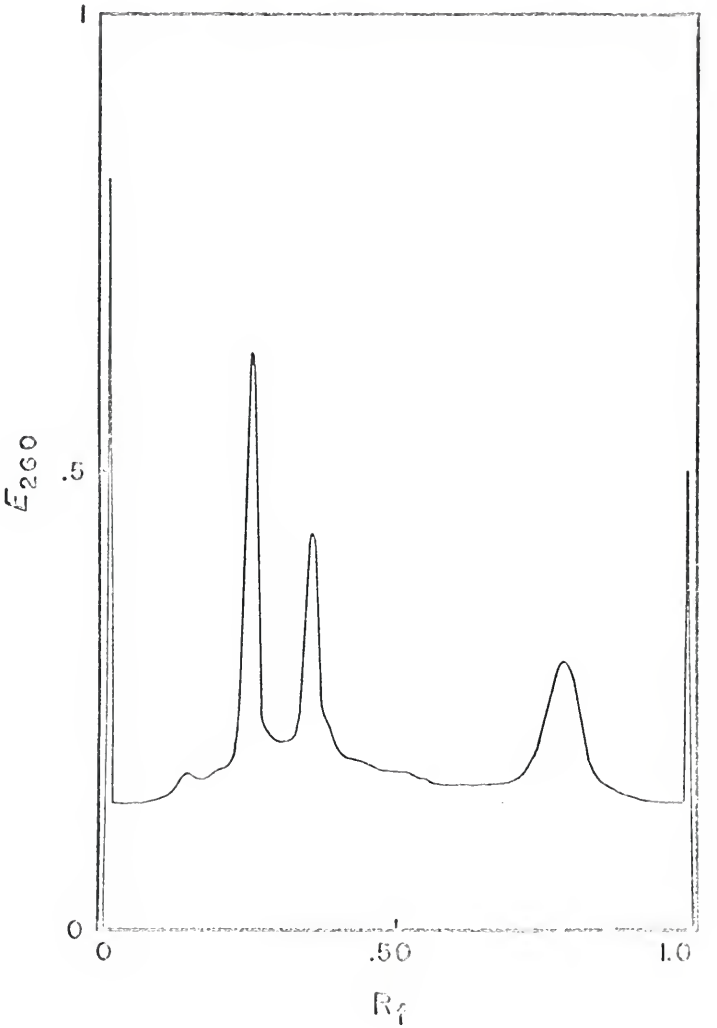


Fig. 19. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Pineapple' orange fruits harvested 11-25-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

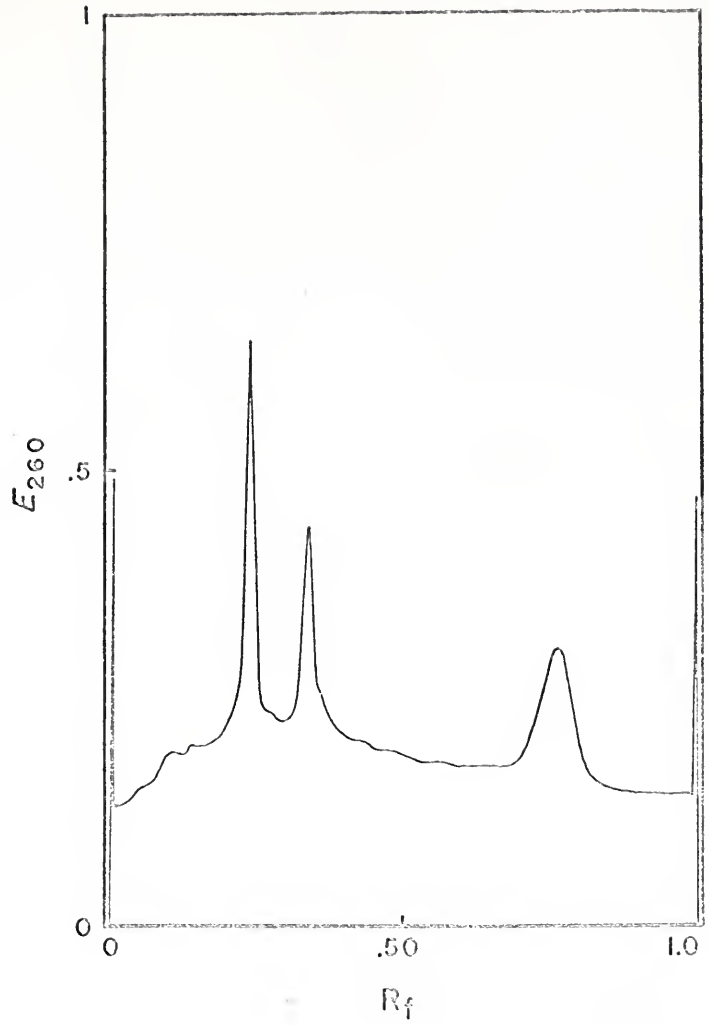


Fig. 20. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Pineapple' orange fruits harvested 12-22-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

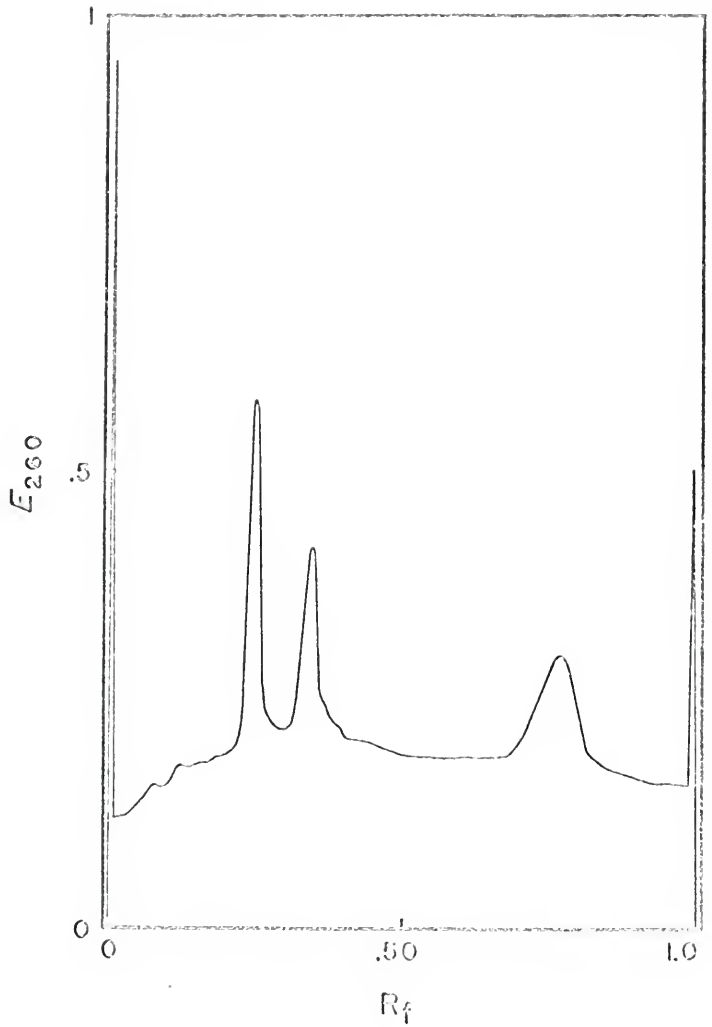


Fig. 21. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Pineapple' orange fruits harvested 1-17-71. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

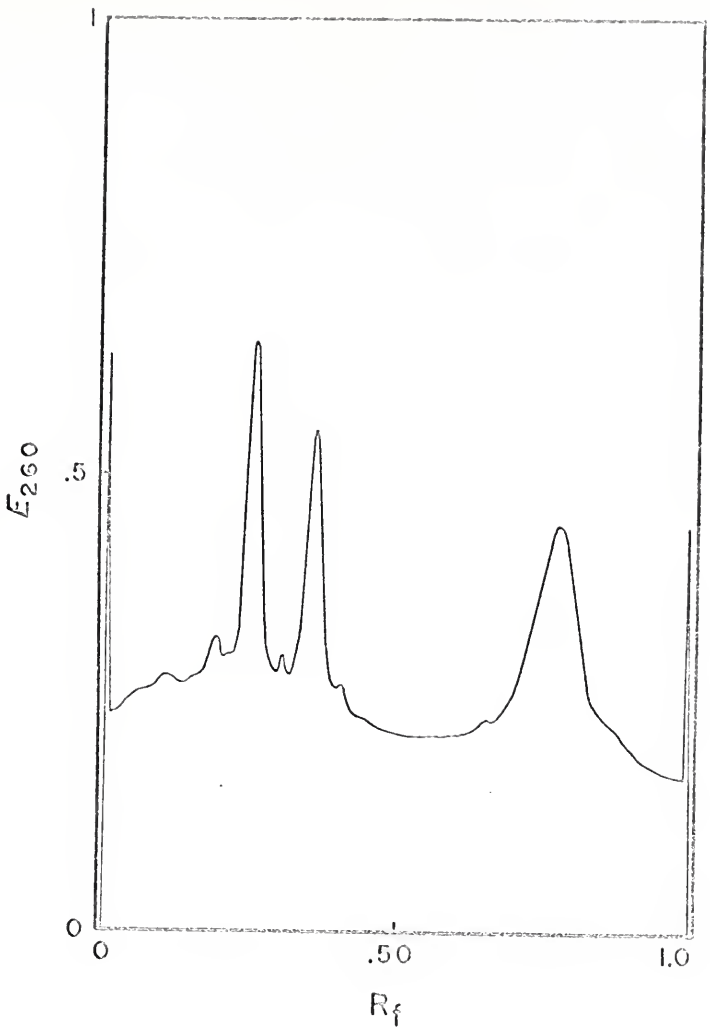


Fig. 22. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Parson Brown' orange fruits harvested 7-23-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

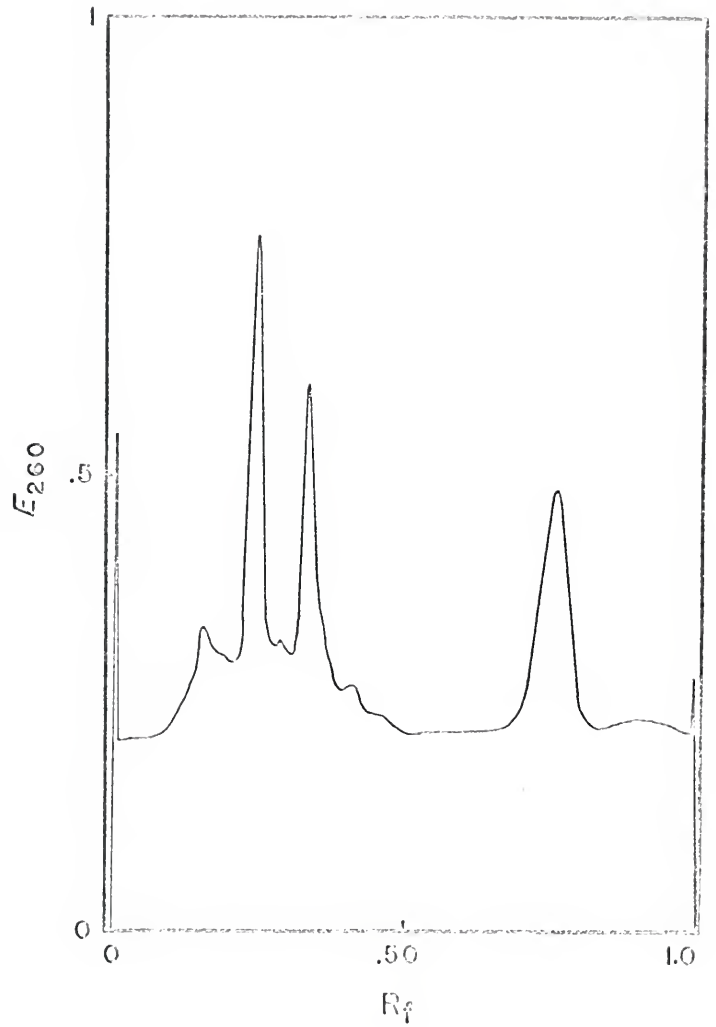


Fig. 23. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Parson Brown' orange fruits harvested 9-26-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

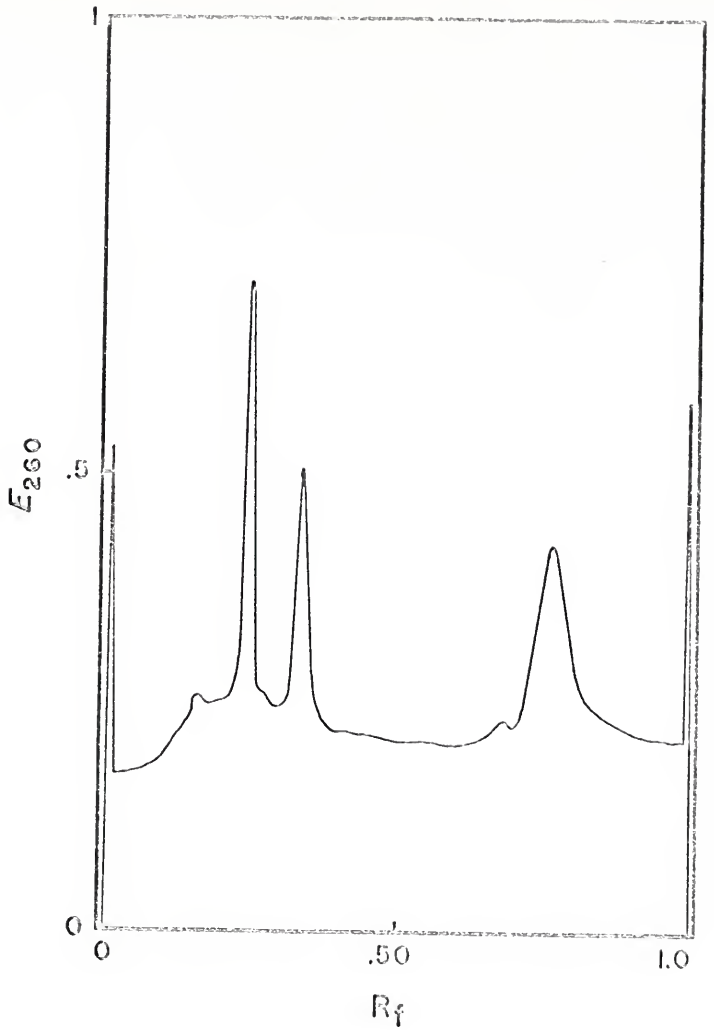


Fig. 24. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Parson Brown' orange fruits harvested 11-25-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

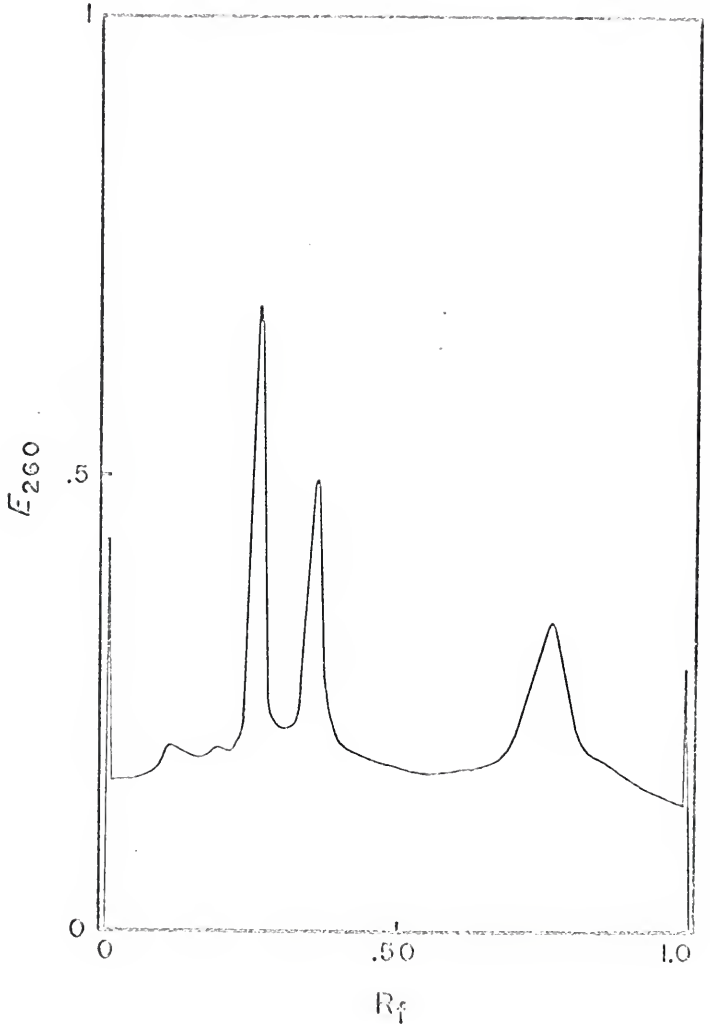


Fig. 25. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Parson Brown' orange fruits harvested 12-22-70. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

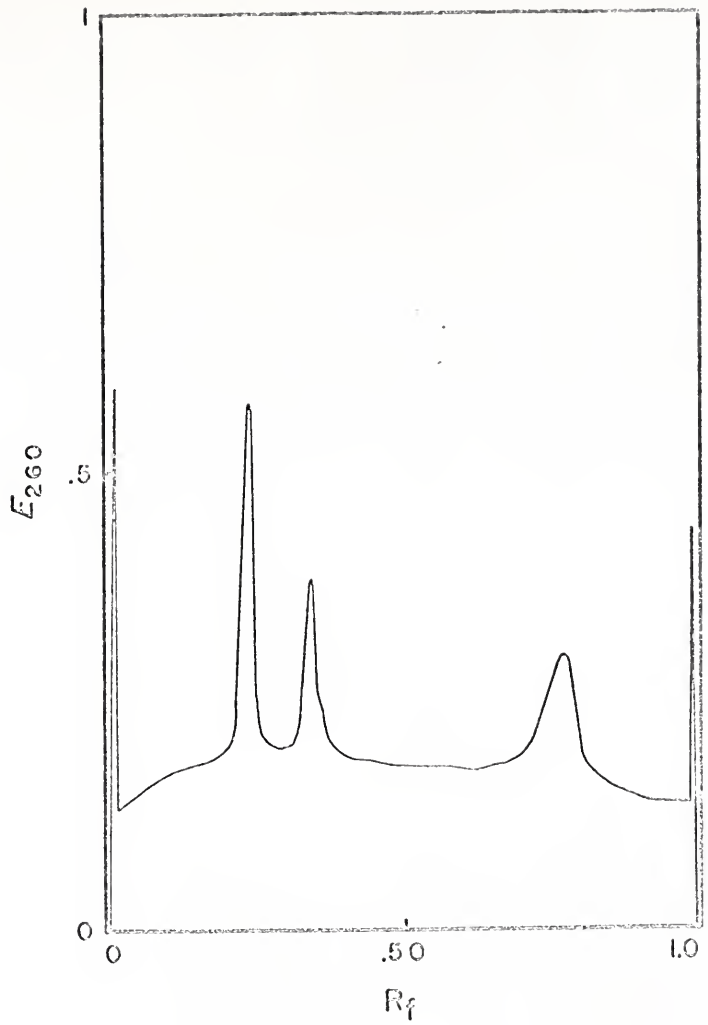


Fig. 26. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from 'Parson Brown' orange fruits harvested 1-17-71. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel.

of the cytoplasmic rRNA subunits, 25S:18S, is usually 1.8-2.0 in various types of tissue. Ratios deviating from this would indicate an increase in the percent turnover of RNA (47, 103). The ratios of the major polymeric RNA fractions are presented in Table 6. The trends of the cytoplasmic rRNA subunit ratios were similar in fruits of 'Hamlin', 'Pineapple', and 'Parson Brown'. The ratios increased with fruit growth from 1.25 to 1.66 in 'Hamlin' fruit, 1.00 to 1.78 in 'Pineapple' fruit, and 1.00 to 2.00 in 'Parson Brown' fruit. However, in 'Orlando' tangelo fruit, the ratios of the same subunit fruits did not show an increase until December 12, 1970, at which time the ratio was 1.6. The ratios of the rRNA:sRNA followed the same trend as the rRNA subunits for each variety. These data suggests that the percent turnover of cytoplasmic RNA decreases with growth becoming rather constant at the completion of stage II of fruit growth.

Labeling of 'Calamondin' Fruit with ^{32}P at Various Stages of Growth and Development

To ascertain further the nature of the nucleic acid system, ^{32}P was applied to fruit at 3 different stages of growth and development. Preferential synthesis of specific RNA fractions has been shown to occur concomitantly with changes in the metabolism of various plant organs (50, 77). 'Calamondin' fruit of 3 physiological ages were labelled with ^{32}P applied through the stem end for 24 hr. 'Calamondin' fruit were chosen as experimental material since all 3 ages could be obtained from the same plant at the same time. The RNA was fractionated by gel-electrophoresis and the ^{32}P activity in the various regions of the gel determined by scintillation counting. The electrophoretic patterns of ^{32}P incorporation into RNA of fruits at different physiological ages

Table 6. Ratios of the cytoplasmic RNA fractions from citrus fruit pulp at various stages of growth and development

Harvest Date	25S:18S	rRNA:sRNA	Harvest Date	25S:18S	rRNA:sRNA
'Orlando' tangelo			'Hamlin' orange		
7-23-70	1.02	1.02	7-23-70	1.25	0.64
9-26-70	1.00	1.00	9-11-70	1.38	1.41
11-25-70	1.22	1.11	11-25-70	1.62	1.57
12-12-70	1.60	1.33	12-22-70	1.66	1.67
'Pineapple' orange			'Parson Brown' orange		
7-23-70	1.00	0.60	7-23-70	1.00	0.89
9-26-70	1.41	1.39	9-26-70	1.09	1.22
11-25-70	1.79	1.49	11-25-70	1.57	1.28
12-22-70	1.78	1.45	12-22-70	1.57	1.28
1-17-71	1.68	1.57	1-17-71	2.00	1.62

are presented in Figs. 27, 28, and 29. The major UV absorbing RNA components were cytoplasmic rRNA and sRNA. It must be pointed out that counting of 2 mm gel slices did not provide a radioactive profile with the same degree of resolution found in the UV absorbance scans. Incorporation of ^{32}P occurred in young fruits and fruits having completed their increase in size but still green. The most noticeable feature of these radioactive profiles is the high rate of incorporation by green fruits which had completed growth and the absence of ^{32}P incorporation by mature fruit prior to abscission. In the electrophoretograms of young fruit, ^{32}P activity was found in 3 regions, rRNA, mRNA, and sRNA. Maximum activity in these regions were 1,250 cpm, 1,470 cpm, and 1,550 cpm, respectively. The activity in the mRNA region of the electrophoretogram is of special interest. This particular RNA species has been suggested as being a DNA-RNA complex (48, 49, 50), and is an important intermediate in protein synthesis (44).

Separation of Acid-Soluble Nucleotides in Citrus Fruit Pulp at Various Stages of Growth and Development

Much evidence has been accumulated indicating the importance of nucleotides in plant metabolism, particularly carbohydrate metabolism. Therefore, the fruit pulp was examined for nucleotide content. With some minor changes, extraction and analyses were similar to those used for other types of tissues (18, 90). A typical ion-exchange chromatogram of the 5' mononucleotides is shown in Fig. 30. Overlapping of several of the nucleotides, AMP-CDP, CTP-GDP, and UDP-ATP, is a common occurrence with this type of analysis. The elution sequence of the acid-soluble nucleotides in the fruit pulp of the 4 varieties are presented in Figs. 31 through 49. Identification of the various fractions

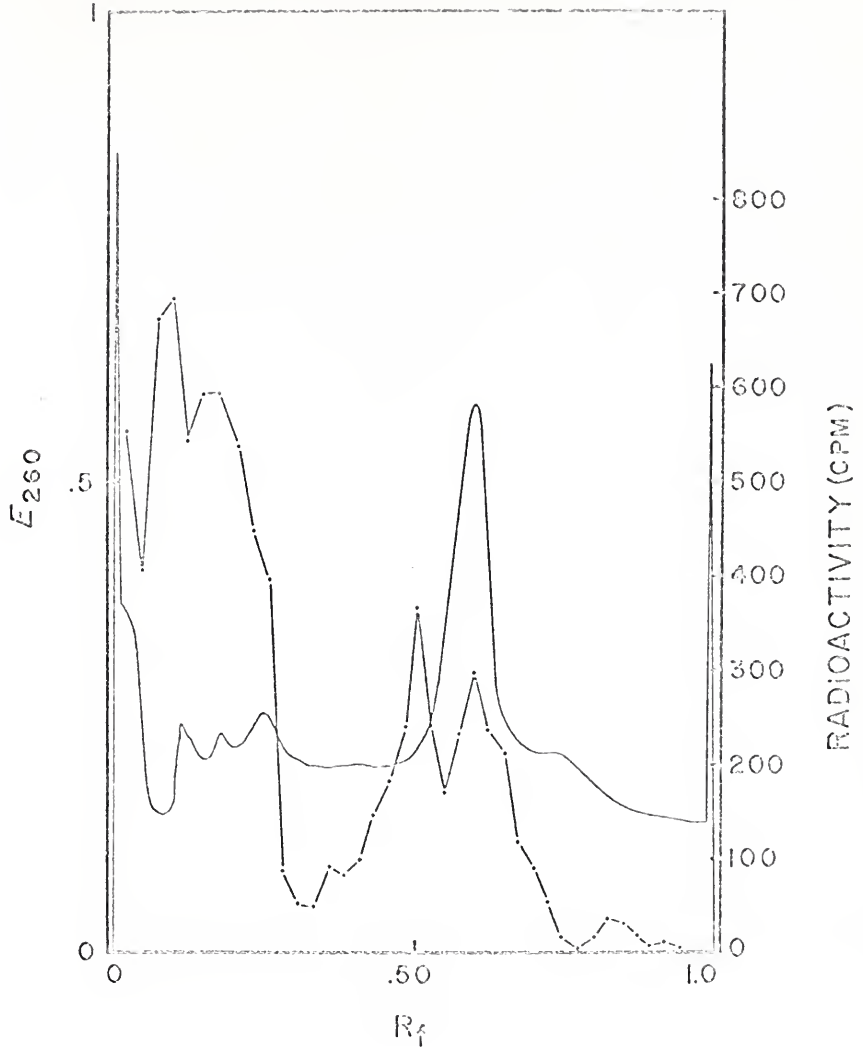


Fig. 27. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from immature 'Calamondin' orange fruit 1.5 cm in diameter. Fruits were labeled with ^{32}P orthophosphate (0.01 mCi/fruit) applied to the stem end for 24 hours. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel. ——, E_{260}; –·–·–, Radioactivity.

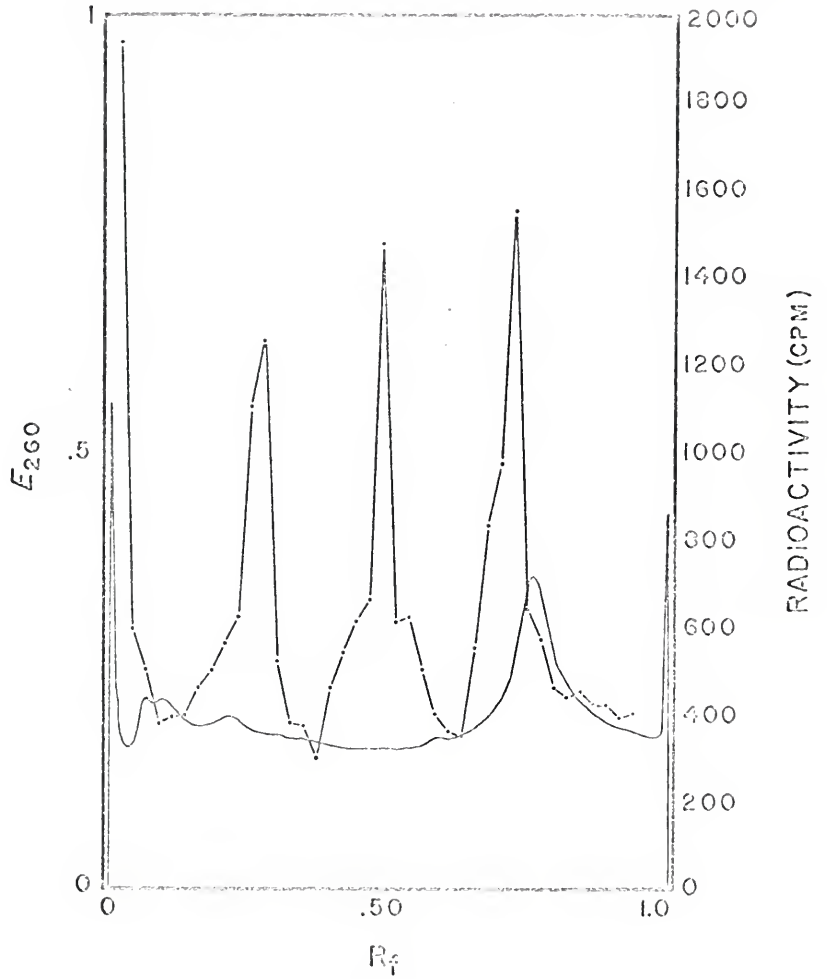


Fig. 28. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from mature, green 'Calamondin' orange fruits 3.0 cm in diameter. Fruits were labeled with ^{32}P orthophosphate (0.01 mCi/fruit) applied to the stem end for 24 hours. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel. ——, E_{260}; —·—·—, Radioactivity.

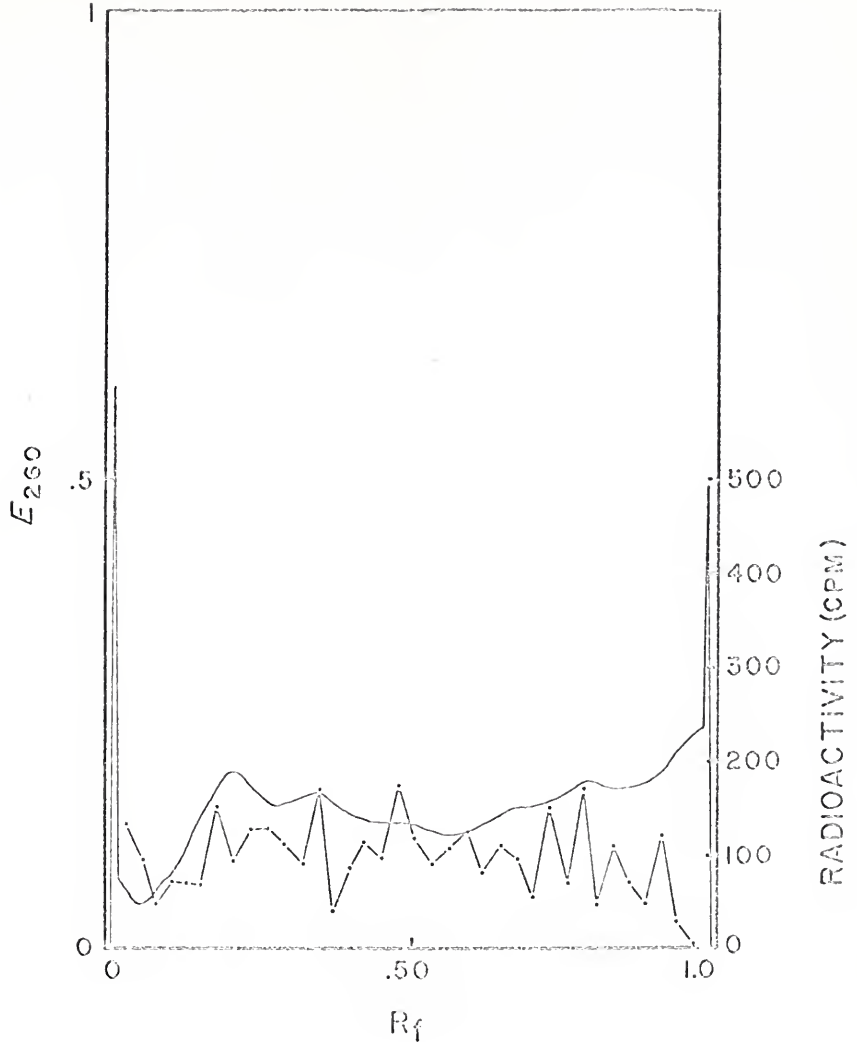


Fig. 29. Electrophoretogram on 2.6% acrylamide gel of cytoplasmic RNA from very mature 'Calamondin' orange fruits. Fruits were labeled with ^{32}P orthophosphate (0.01 mCi/fruit) applied to the stem end for 24 hours. Electrophoresis was carried out for 70 minutes at 5 mAmps/gel. ——, E_{260}; —·—·—, Radioactivity.

Fig. 30. Anion-exchange chromatogram of the 5' mononucleotides separated on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6 N ammonium formate.

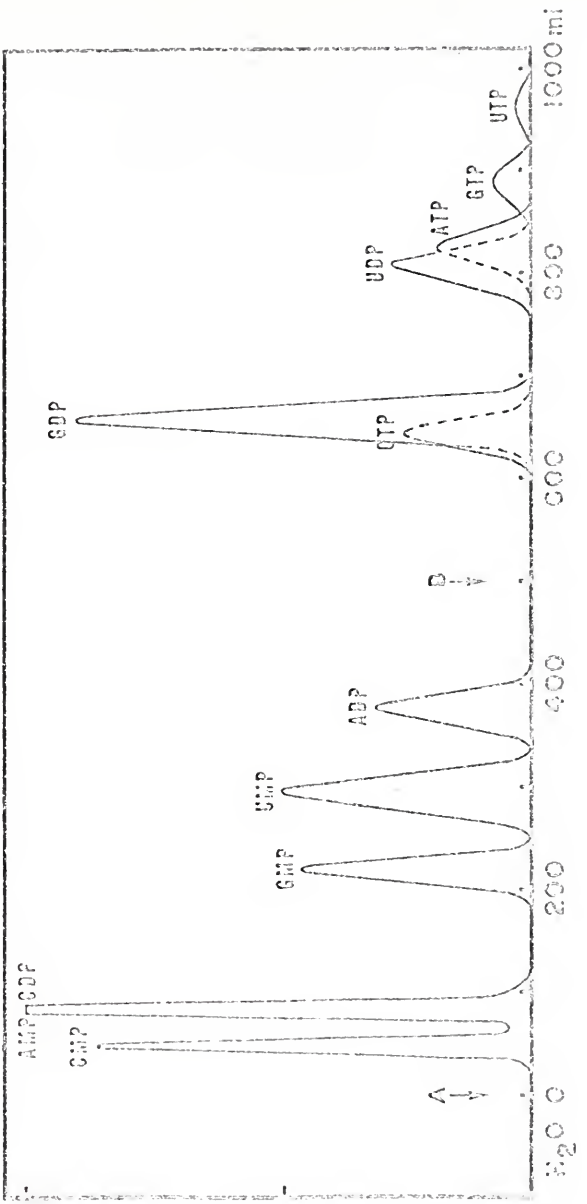

A
B
AMPnGDP
GMP
GMP
GMP
ADP
GDP
GTP
UDP
ATP
GTP
UTP
320 0
200
400
600
800
1000ml
FRACTION
.2
.5
0
A_{260}

Fig. 31. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Orlando' tangelo fruit pulp harvested 8-19-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

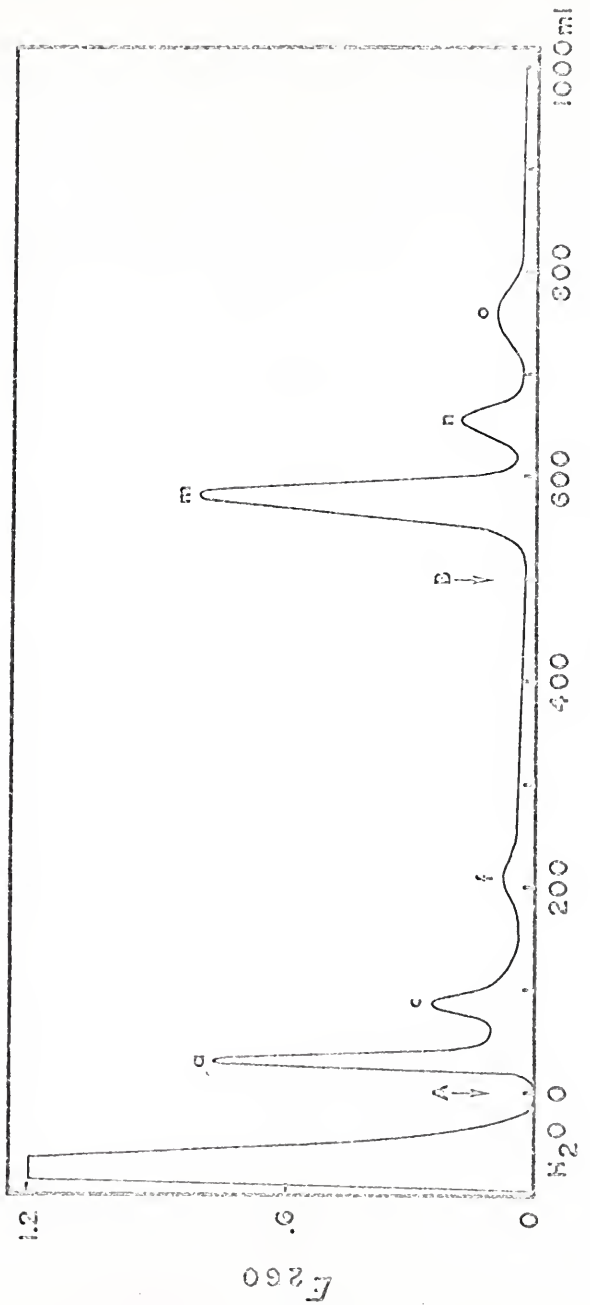

E_{280}
1.2
0.6
0
H_2O 0
200
400
600
800
1000ml
FRACTION
A
B
a
b
c
d
e
f

Fig. 32. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Orlando' tangelo fruit pulp harvested 9-26-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

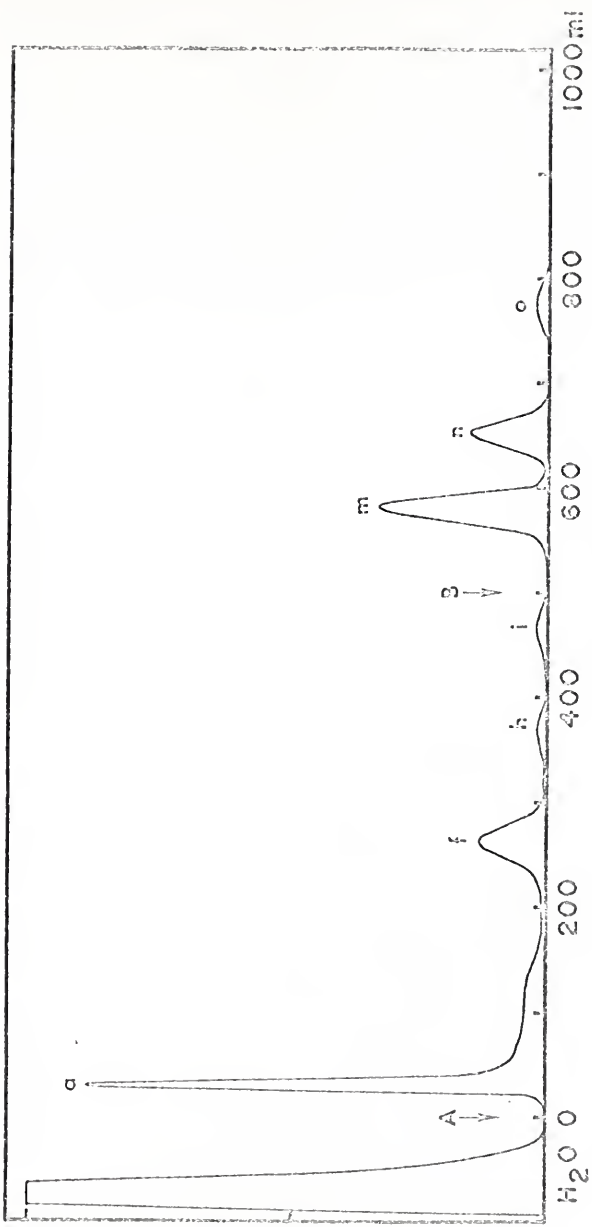

E_{260}
1.2
.6
0
H_2O 0
A
a
200
f
400
h
i
B
600
m
n
o
800
1000ml
FRACTION

Fig. 33. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Orlando' tangelo fruit pulp harvested 11-25-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

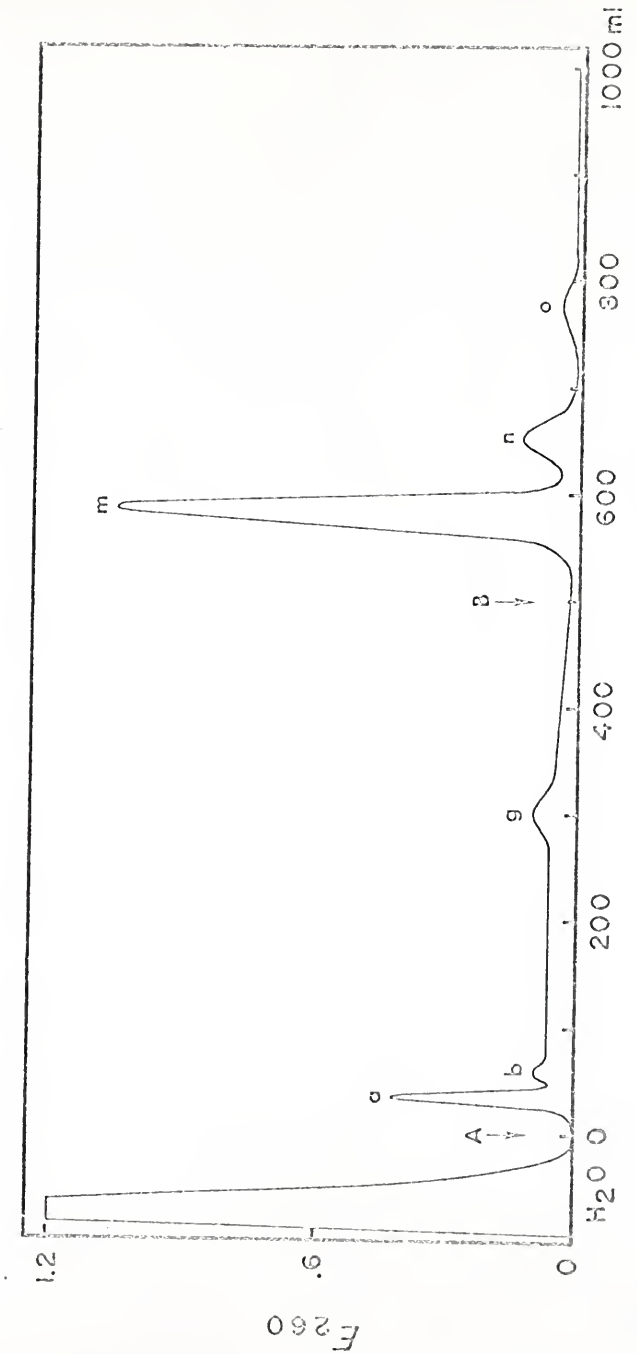

1.2
.6
0
E_{260}
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
B
a
b
g
m
n
o

Fig. 34. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Orlando' tangelo fruit pulp harvested 12-12-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

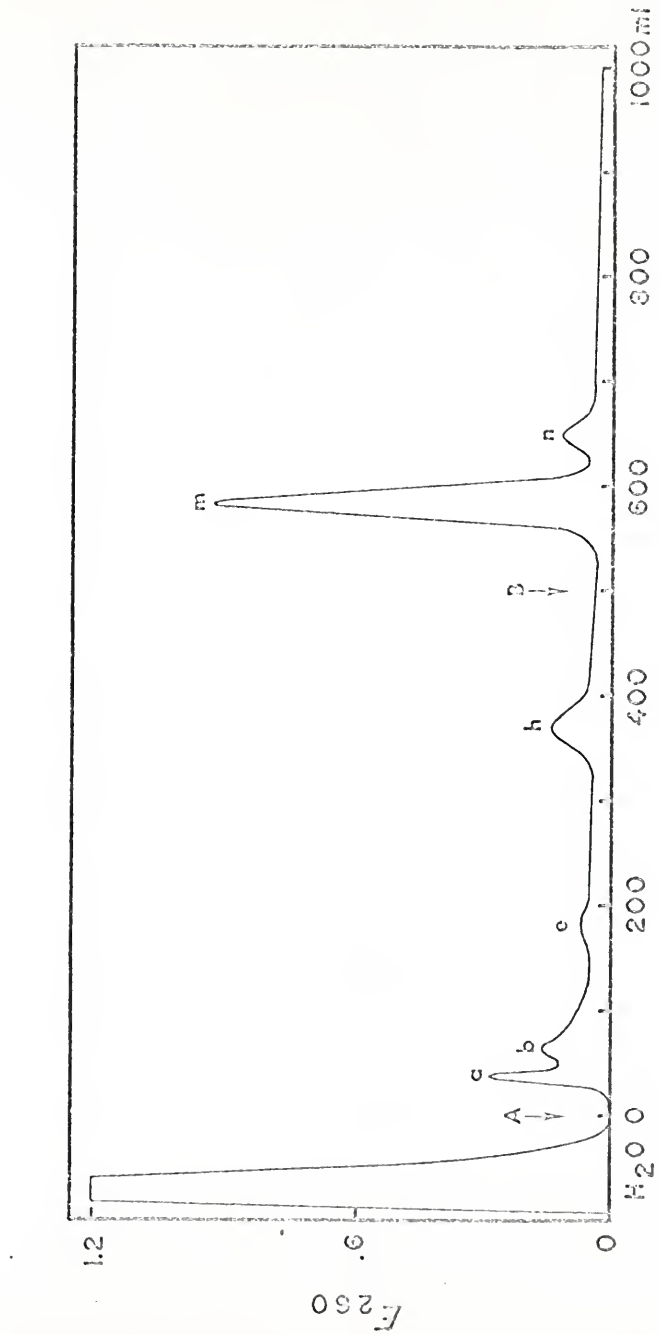

1.2
.6
0
A_{250}
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
B
a
b
c
e
h
m
n

Fig. 35. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Hamlin' orange fruit pulp harvested 7-23-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

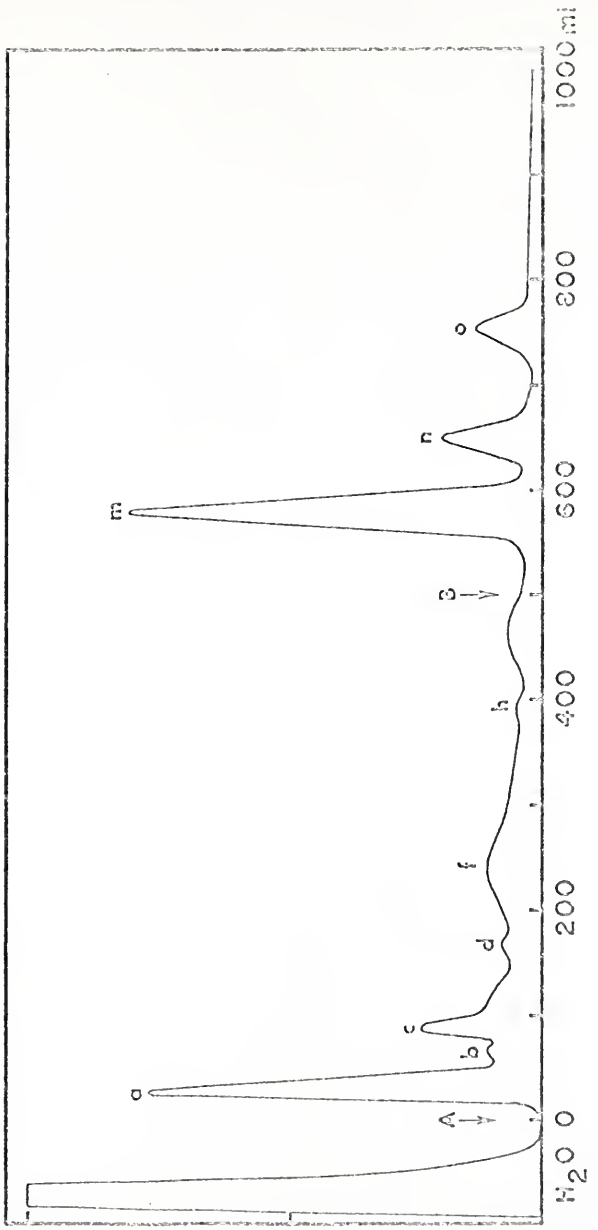

1.2
0.6
0
E_{260}
H_2O
0
200
400
600
800
1000 ml
FRACTION
A
a
b
c
d
f
g
h
m
n
o

Fig. 36. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Hamlin' orange fruit pulp harvested 8-19-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

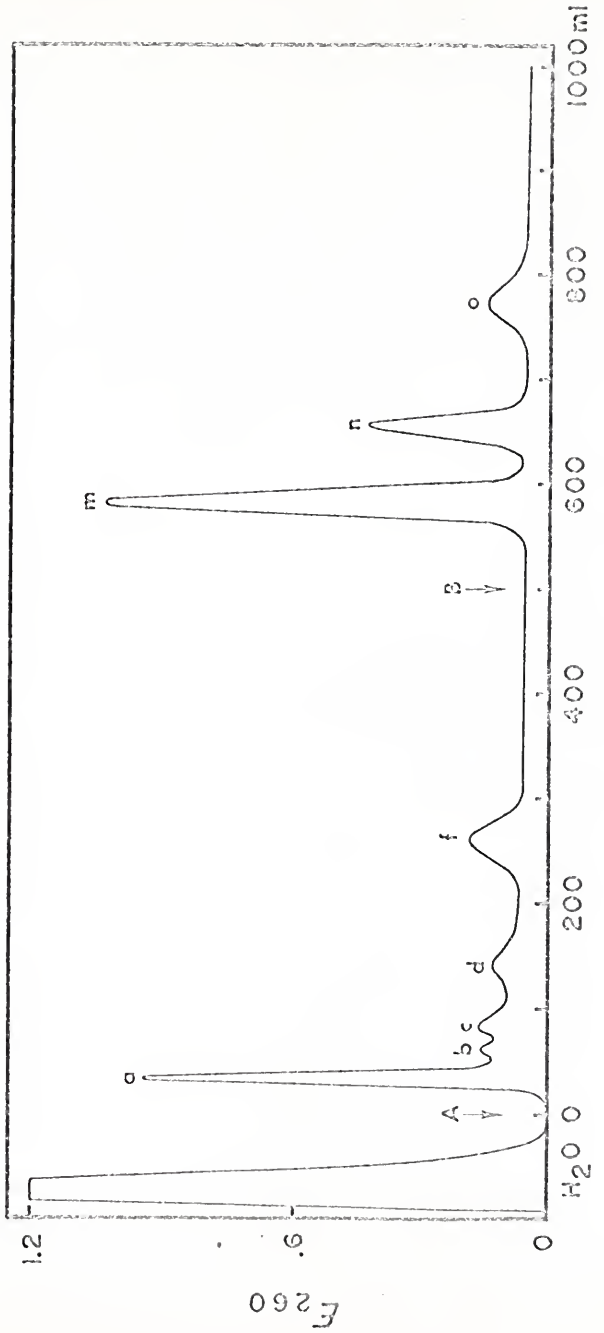

E_{260}
1.2
.6
0
H_2O 0
A
a
b c
d
f
B
m
n
o
200
400
600
800
1000 ml
FRACTION

Fig. 37. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Hamlin' orange fruit pulp harvested 9-11-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

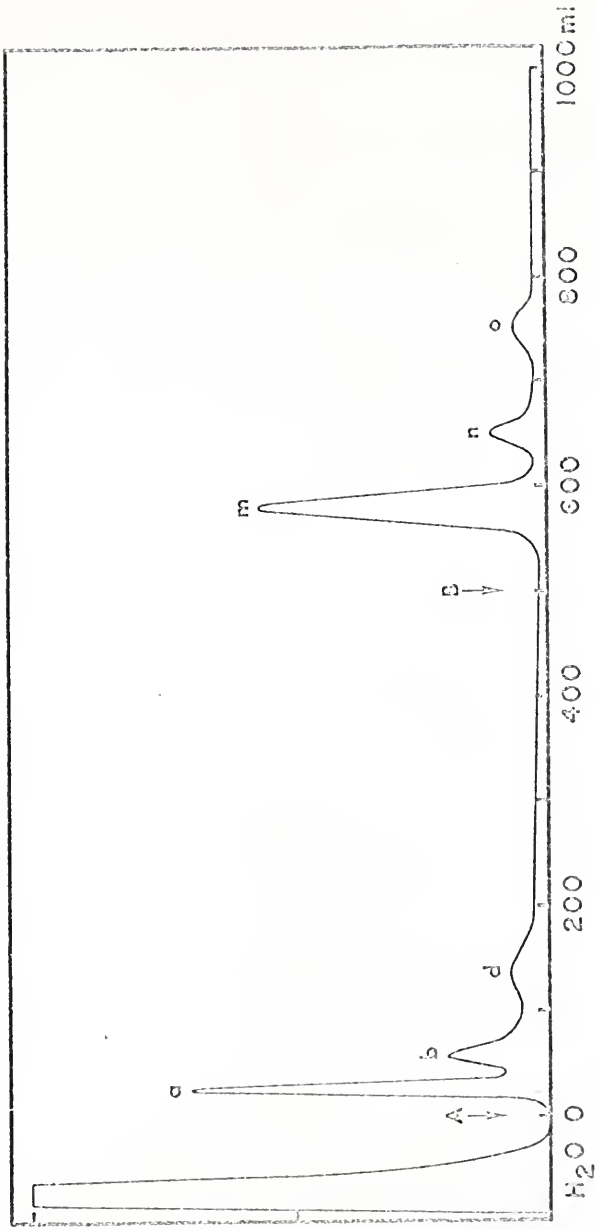

1.2
.6
0
E_{280}
H_2O 0
200
400
600
800
1000ml
FRACTION
A
B
a
b
c
d
m
n
o

Fig. 38. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Hamlin' orange fruit pulp harvested 11-25-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

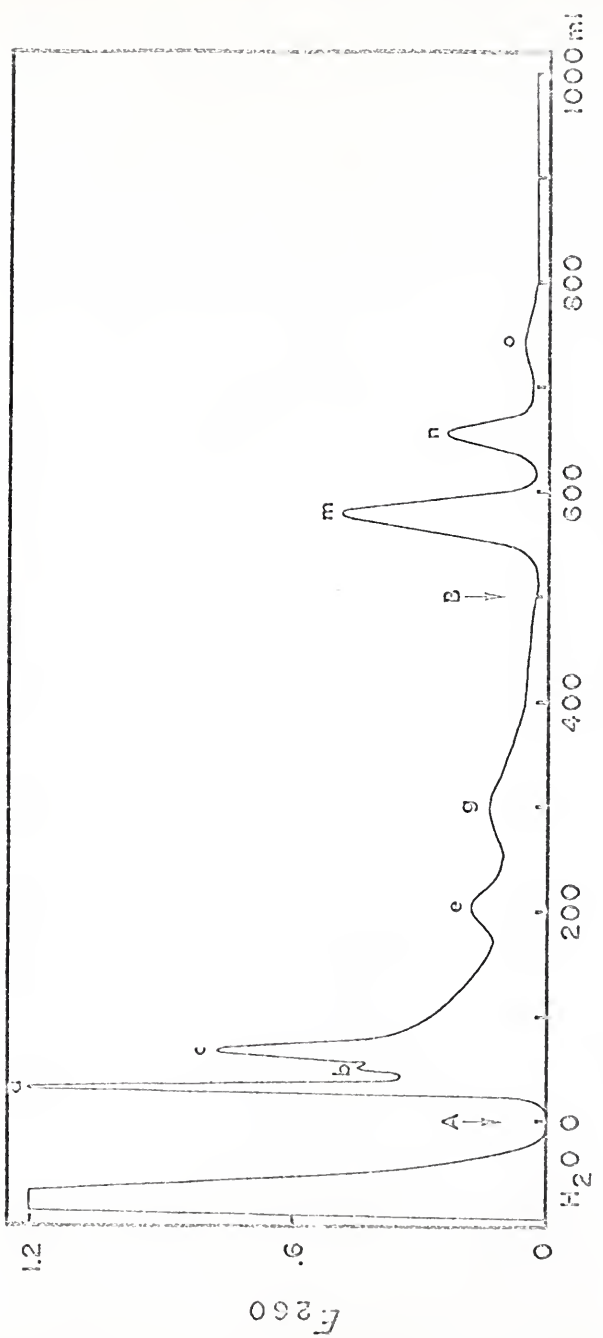

f_{260}
1.2
.6
0
H_2O
0
200
400
600
800
1000 ml
FRACTION
A
B
a
b
c
d
e
g
m
n
o

Fig. 39. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Hamlin' orange fruit pulp harvested 12-22-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

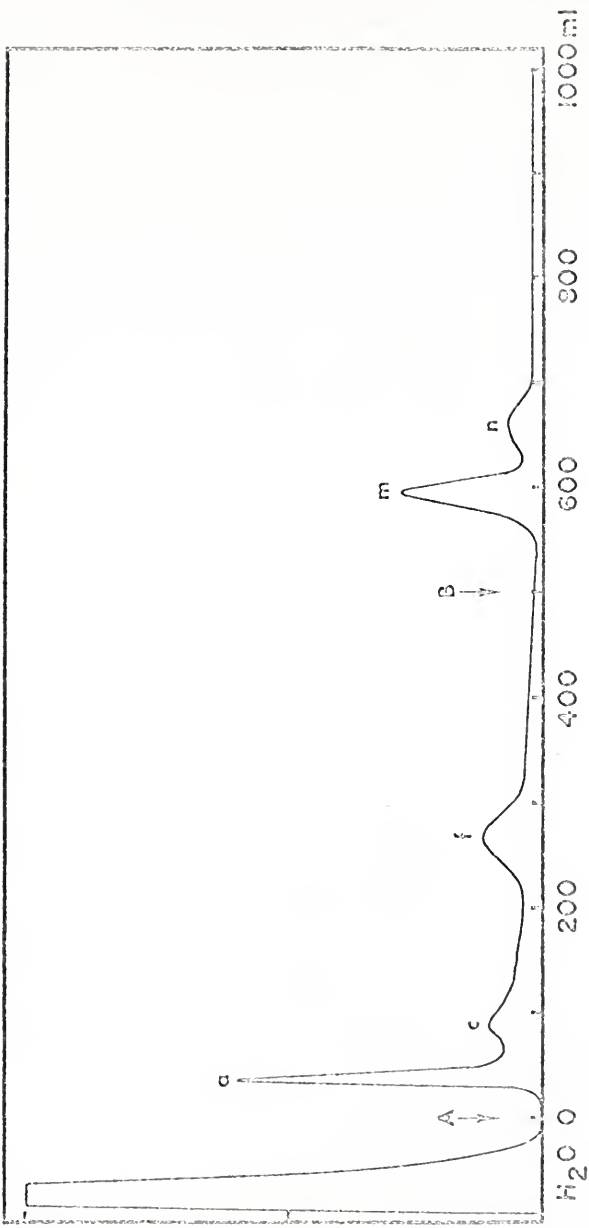

E_{280}
1.2
0.6
0
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
B
a
c
f
m
n

Fig. 40. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Pineapple' orange fruit pulp harvested 8-19-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

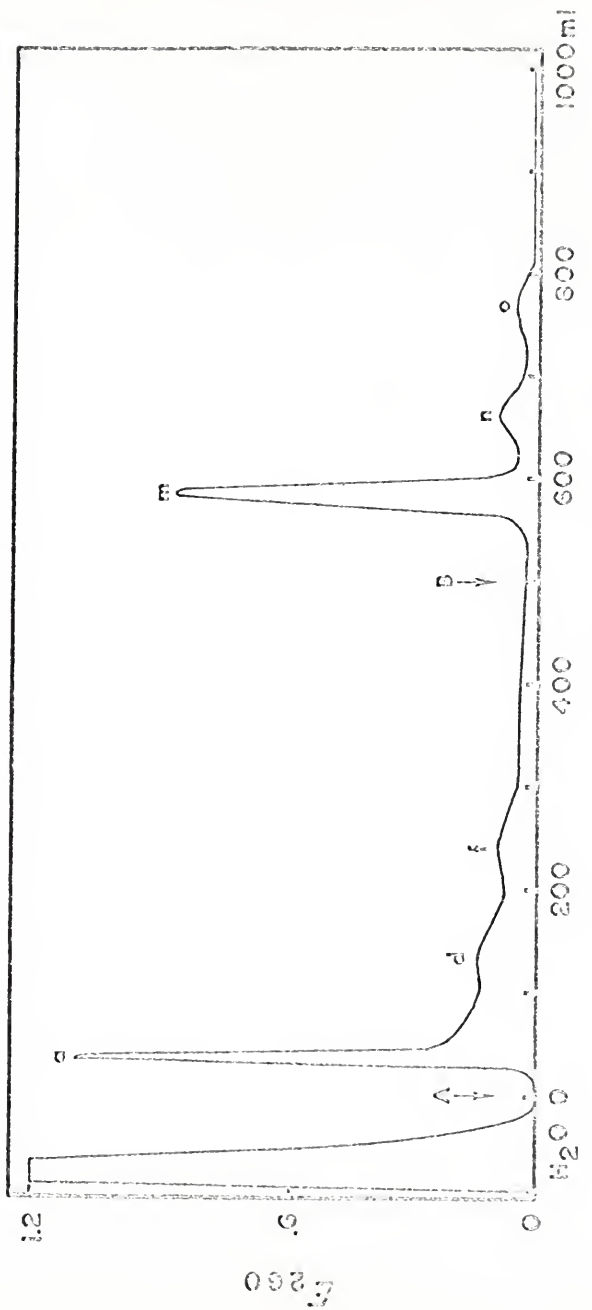

ε_{260}
1.2
0.6
0
H_2O
A
B
C
D
E
a
b
c
d
e
f
g
0
200
400
600
800
1000 ml
FRACTION

Fig. 41. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Pineapple' orange fruit pulp harvested 9-26-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

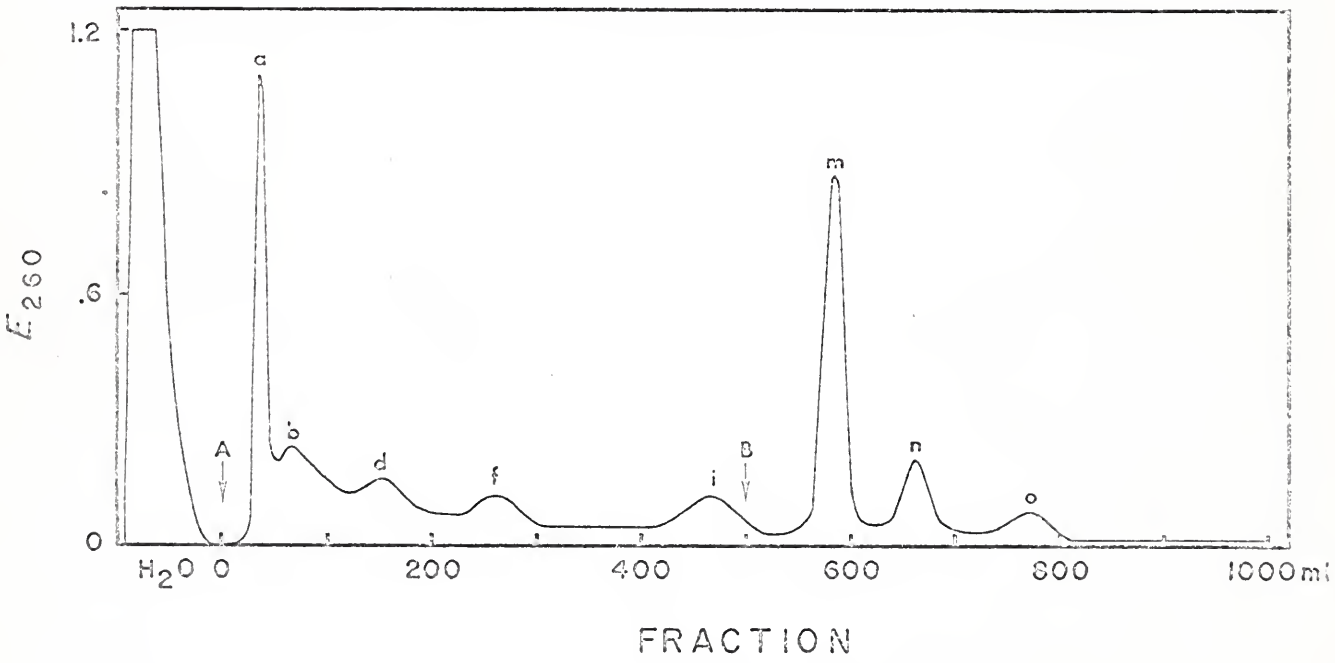

1.2
.6
0
E_{260}
a
b
d
f
i
m
n
o
A
B
H_2O 0
200
400
600
800
1000 ml
FRACTION

Fig. 42. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Pineapple' orange fruit pulp harvested 11-25-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

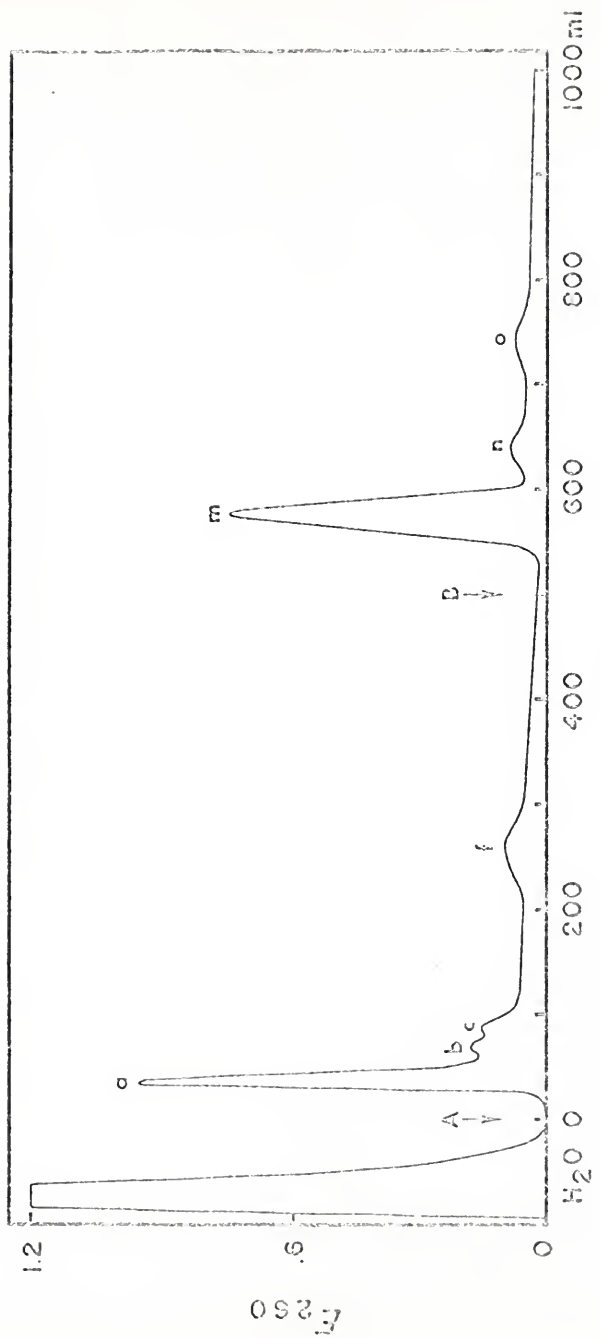

1.2
.6
0
E_{280}
H_2O 0
200
400
600
800
1000ml
FRACTION
A
B
a
b
c
f
m
n
o

Fig. 43. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Pineapple' orange fruit pulp harvested 12-22-70. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

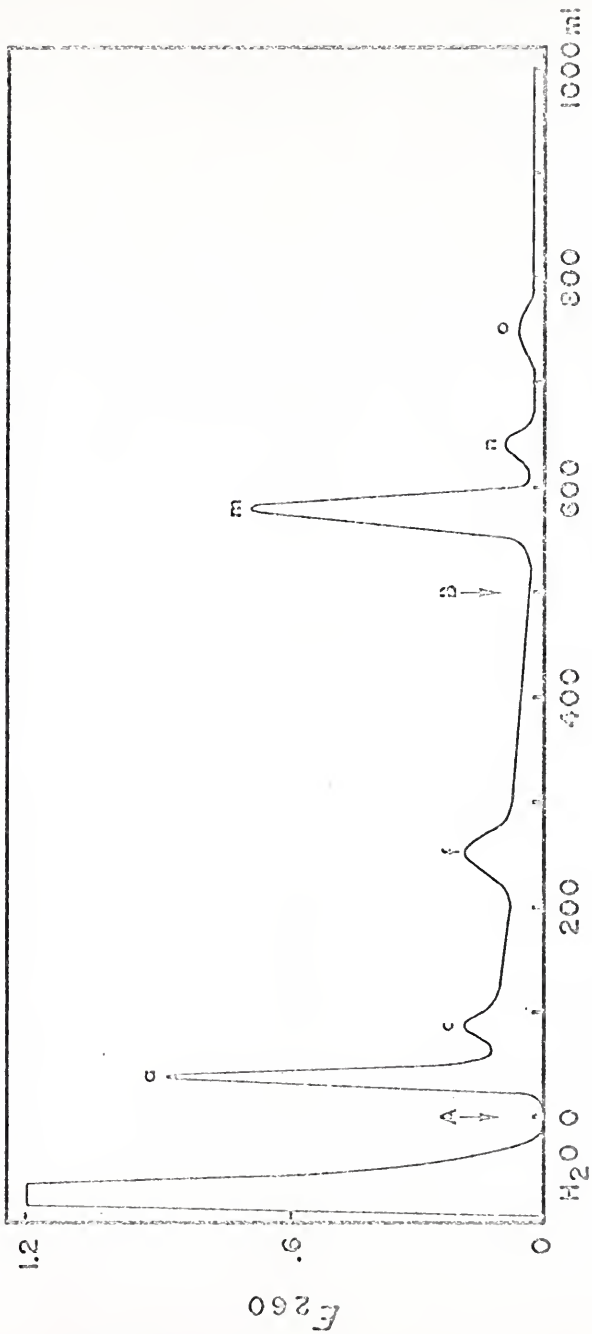

E_{260}
1.2
.6
0
H_2O 0
A
a
c
f
B
m
n
o
200
400
600
800
1000 ml
FRACTION

Fig. 44. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Pineapple' orange fruit pulp harvested 1-17-71. Separation of the extract was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

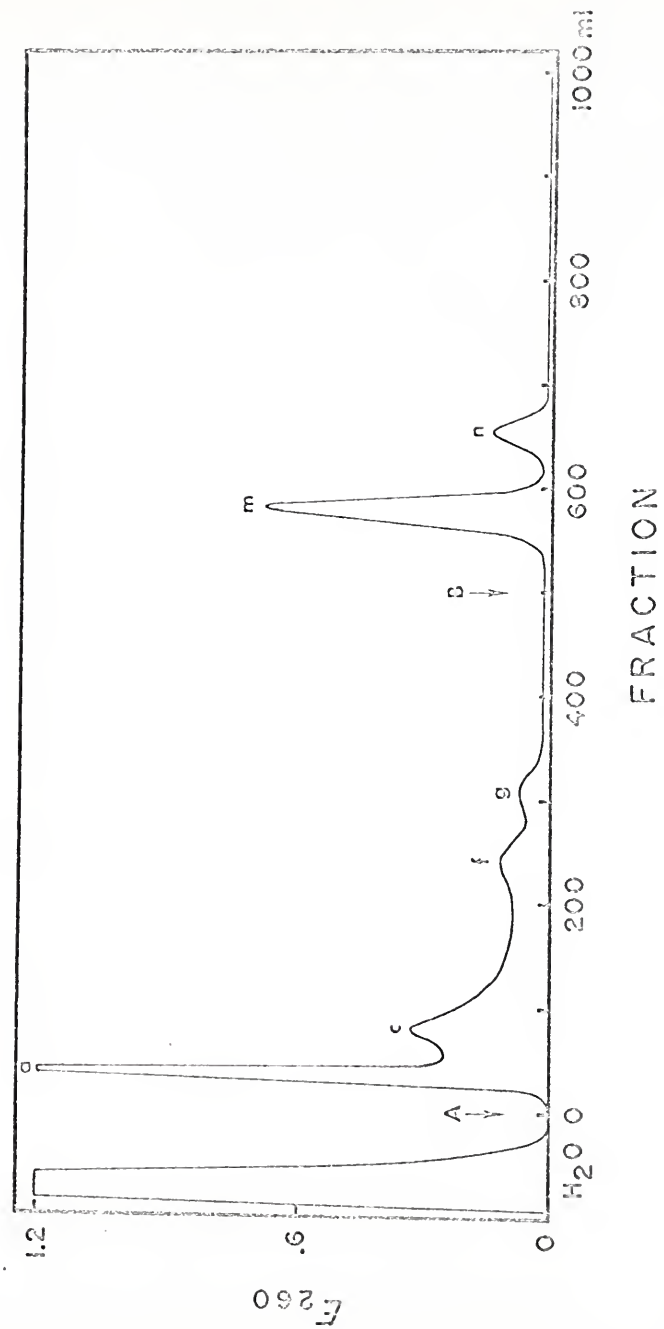

1.2
.6
0
E_{280}
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
B
C
D
E
F
G
H

Fig. 45. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Parson Brown' orange fruit pulp harvested 8-19-70. Separation was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

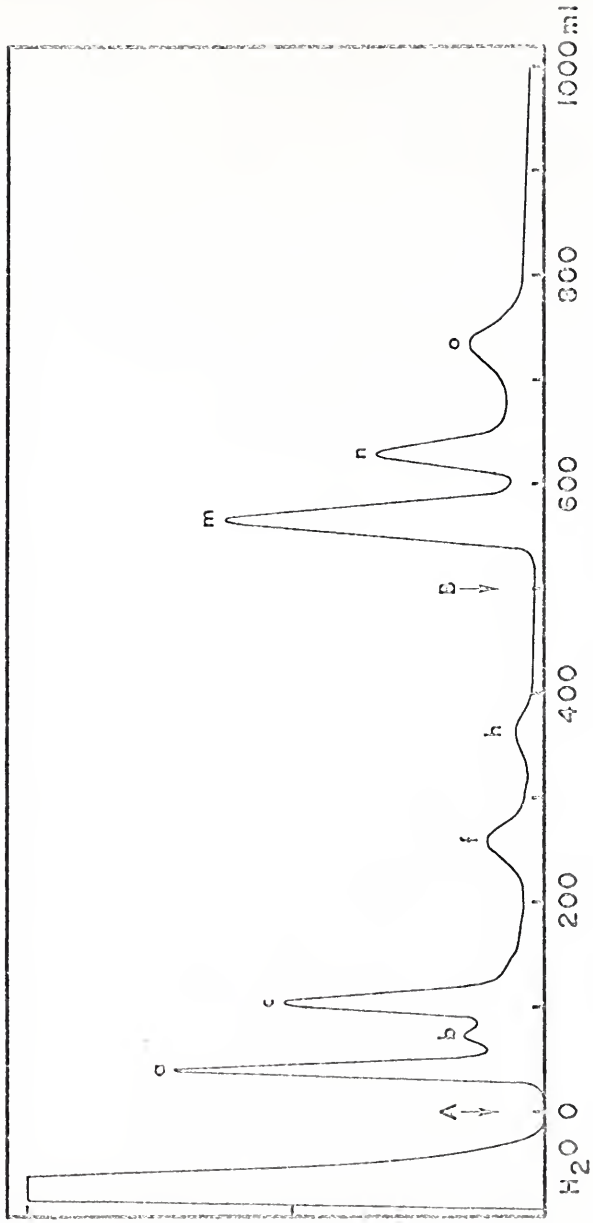

1.2
0.6
0
F_{260}
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
B
a
b
c
f
h
m
n
o

Fig. 46. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Parson Brown' orange fruit pulp harvested 9-26-70. Separation was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

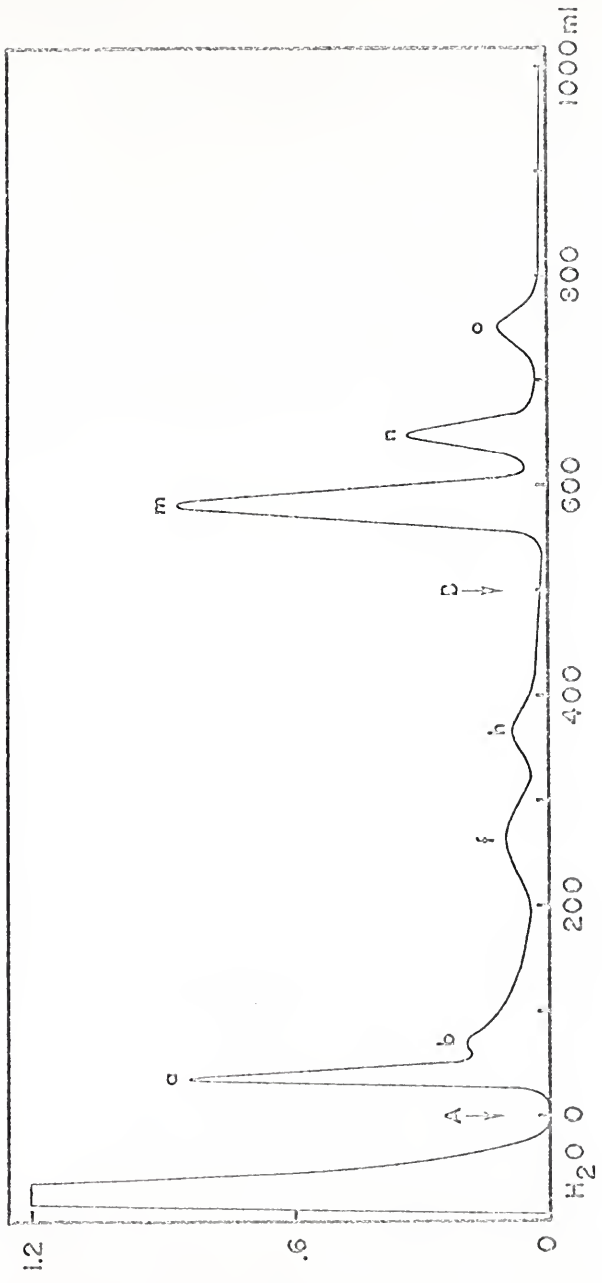

A_{260}
1.2
.6
0
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
a
b
f
h
D
m
n
o

Fig. 47. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Parson Brown' orange fruit pulp harvested 11-25-70. Separation was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

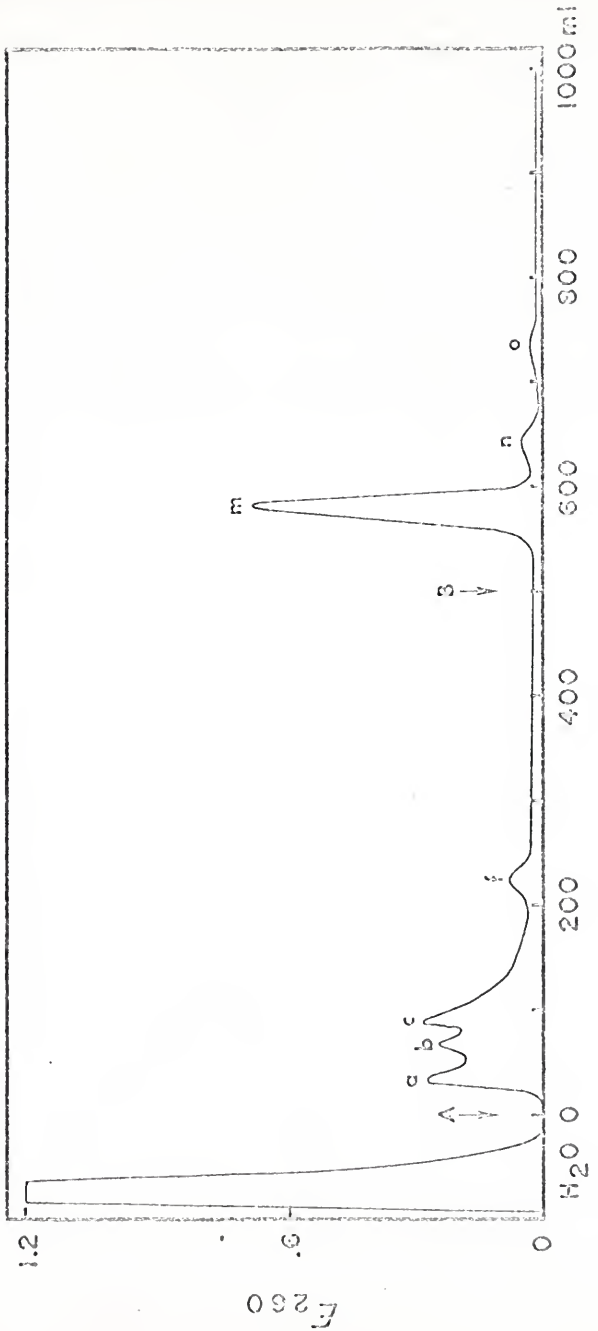

E_{280}
1.2
0.6
0
H_2O 0
200
400
600
800
1000 ml
FRACTION
A
a
b
c
d
B
m
n
o

Fig. 48. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Parson Brown' orange fruit pulp harvested 12-22-70. Separation was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.

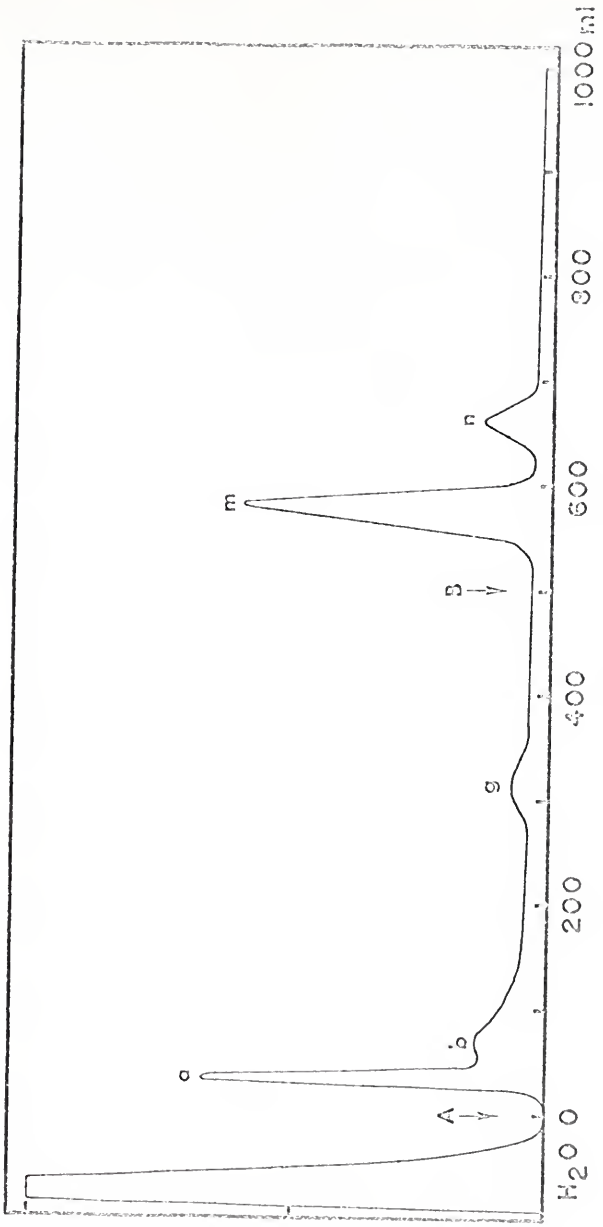
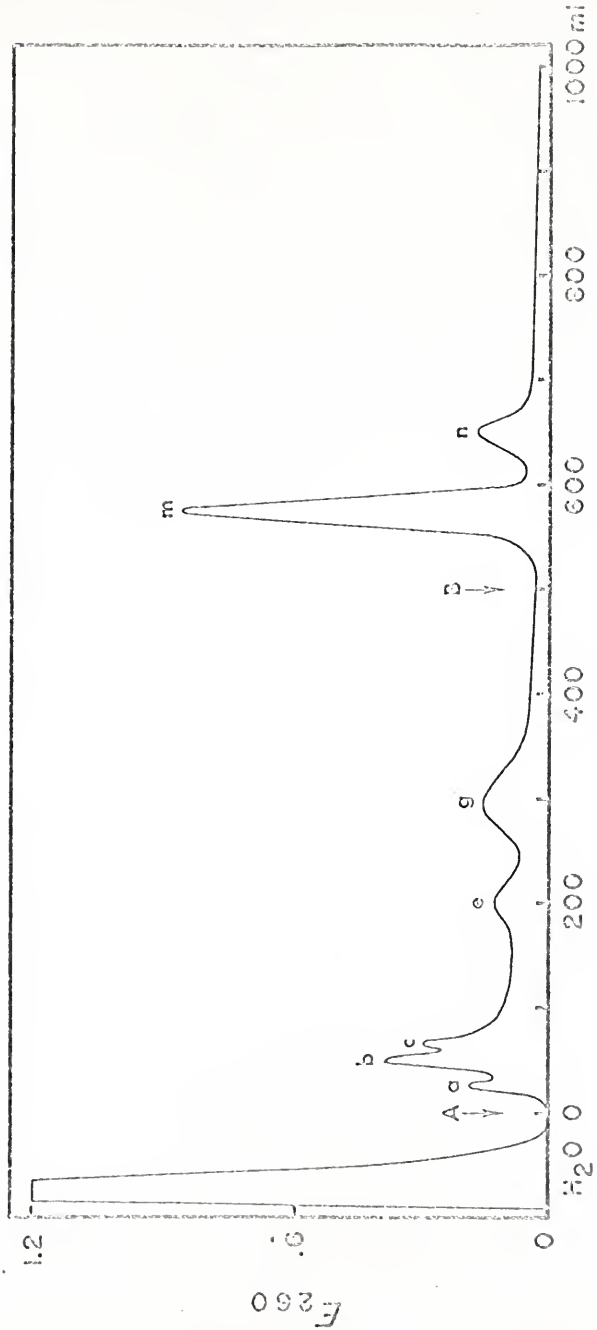

E_{280}
1.2
.6
0
H_2O
A
a
b
g
B
m
n
200
400
600
800
1000 ml
FRACTION

Fig. 49. Anion-exchange chromatogram of the acid-soluble nucleotides of 'Parson Brown' orange fruit pulp harvested 1-17-71. Separation was on a Dowex 1 X 8 (formate form) ion-exchanger in a formate system. A: water to 4N formic acid; B: 4N formic acid to 1.6N ammonium formate.


1.2
.6
0
E_{260}
H_2O 0
200
400
600
800
1000ml
FRACTION
A
a
b
c
d
e
g
m
n

was based on their relative position of elution, 280:260 and 250:260 nm ratios and Emax (Tables 7 and 8), and TLC analysis where possible. The presence of residues from the elution solvents interfered with TLC analysis of many of the fractions. The position of elution matched very closely the position of several standard nucleotides. However, comparison of their absorbance ratios and Emax to those of the standards indicate they are not the same compounds. Also, the Rfs of several of the major fractions from TLC analysis did not correspond to any of the Rfs for the standards. However, the presence of other UV absorbing compounds can cause a shift in the UV absorption spectrum. Tentative identification of several of the fractions based on elution position from the column is presented in Table 8.

Quantitative estimations of these fractions were made by comparing the area under each peak to that of a known nucleotide having approximately the same Emax and elution position. These data are presented in Table 9. There does not appear to be any specific trend in the amount of either the individual fractions or total nucleotides during growth and development. The total concentrations in mg/40 g of pulp ranged from 0.969 mg to 1.029 mg in 'Orlando' tangelo fruit, 0.779 mg to 1.269 mg in 'Hamlin' fruit, 1.082 mg to 1.685 mg in 'Pineapple' fruit and 0.587 mg to 1.926 mg in 'Parson Brown' fruit. The individual fractions comprising the largest percent of the total amount were a, n, and m. It is also interesting to note that these same fractions were present in all fruits analyzed.

Concentration of ATP in Citrus Pulp at Various Stages of Fruit Growth and Development

The concentration of ATP in fruit pulp is most often below the

Table 7. Spectroscopic analysis of fractions of a known mixture of 5' mononucleotides separated on a Dowex 1 X 8 (formate form) ion-exchange in a formate system

Nucleotide	pH	Ratio 280:260	Ratio 250:260	Emax[a]
CMP	3.0	1.796	0.405	279
AMP	2.8	0.283	0.814	260
CDP	2.6	1.893	0.137	280
GMP	2.3	0.694	0.862	257
UMP	2.2	0.351	0.589	260
ADP	2.2	0.227	0.753	258
CTP	2.8	1.465	0.844	278
GDP	2.8	0.558	1.157	255
UDP	3.4	0.257	1.050	262
ATP	3.3	0.422	1.042	252
GTP	3.5	0.352	0.995	254

[a] Wavelength of maximum absorbance in nm.

Table 8. Spectroscopic analysis and tentative identification of the acid-soluble nucleotide fractions extracted from citrus fruit pulp separated on a Dowex 1 X 8 (formate form) ion-exchange in a formate system

Fraction	pH	Ratio 280:260	Ratio 250:260	Emax[a]	Nucleotide
a	3.0	.845	0.637	268	CMP-NAD
b	2.8	.899	0.941	260	AMP
c	2.7	.726	0.973	260	CDP
d	2.5	.419	1.322	256	--
e	--	--	--	--	GMP
f	2.3	.860	0.400	274	--
g	--	--	--	--	UMP
h	--	--	--	--	ADP
m	2.7	.344	0.793	262	--
n	2.8	.450	1.256	263	CTP-GDP
o	2.9	.705	0.532	265	UDP-ATP

[a] Wavelength of maximum absorbance in nm.

Table 9. Estimated quantity of the acid-soluble nucleotide fractions in mg per 40 g of citrus fruit pulp at various stages of growth and development

Harvest Date	Fraction												
	a	b	c	d	e	f	g	h	i	m	n	o	Total
						'Orlando' tangelo							
8-19-70	0.525		0.040		0.018					0.280	0.075	0.030	0.986
9-26-70	0.630					0.090		0.015	0.015	0.175	0.080	0.020	1.025
11-25-70	0.462	0.025					0.040			0.451	0.052	0.020	1.050
12-12-70	0.163	0.308			0.012			0.131		0.373	0.042		1.029
						'Hamlin' orange							
7-23-70	0.722	0.008	0.041	0.014				0.003		0.319	0.116	0.087	1.210
8-19-70	0.643	0.083	0.088	0.082		0.108				0.410	0.238	0.117	1.769
9-11-70	0.429	0.046		0.046						0.263	0.054	0.058	0.896
11-25-70	1.100	0.010	0.225		0.036		0.030			0.250	0.028	0.010	1.689
12-22-70	0.625		0.011							0.102	0.041		0.779

Table 9. Continued

Harvest Date	Fraction												
	a	b	c	d	e	f	g	h	i	m	n	o	Total
						'Pineapple' orange							
8-19-70	0.647		0.447			0.030				0.280	0.076	0.061	1.556
9-26-70	0.789			0.019		0.021				0.189	0.028	0.036	1.082
11-25-70	0.981	0.020	0.009			0.046				0.279	0.034	0.012	1.381
12-22-70	0.528		0.059			0.081				0.330	0.027	0.017	1.042
1-17-71	1.263		0.049			0.032	0.049			0.241	0.051		1.685
						'Parson Brown' orange							
8-19-70	0.762	0.014	0.130			0.057		0.032		0.268	0.146	0.065	1.474
9-26-70	0.753	0.020				0.198				0.362	0.187	0.083	1.603
11-25-70	0.185	0.025	0.054			0.059				0.252	0.012		0.587
12-22-70	0.745	0.095					0.024			0.278	0.040		1.926
1-17-71	0.155	0.080	0.040		0.022		0.049			0.288	0.060		0.694

minimal level of detection with the UV method of analysis, thus requiring the use of an alternate method such as the luciferin-luciferase method described by Buslig and Attaway (24). These results are presented in Table 10. The most noticeable information that can be obtained from these data is the high ATP concentration in the pulp of young fruit, decreasing with fruit growth and development. The ATP concentrations found in this study are similar to those reported by Buslig (23).

Table 10. Concentration of ATP in citrus fruit pulp at various stages of growth and development

Harvest Date	ATP n moles/g pulp	Harvest Date	ATP n moles/g pulp
'Orlando' tangelo		'Hamlin' orange	
7-23-70	146.2	7-23-70	142.5
8-19-70	128.7	8-19-70	93.0
9-26-70	168.3	9-11-70	30.7
10-10-70	98.3	10-10-70	54.4
11-14-70	70.4	11-14-70	42.0
11-25-70	40.6	11-25-70	27.0
12-12-70	50.8	12-12-70	39.3
'Pineapple' orange		'Parson Brown' orange	
7-08-70	152.0	7-08-70	191.4
7-23-70	176.0	7-23-70	230.0
8-19-70	128.5	8-19-70	211.8
9-26-70	62.0	9-26-70	123.7
10-10-70	60.2	10-10-70	78.7
11-14-70	57.3	11-14-70	22.5
11-25-70	26.3	11-25-70	33.0
12-22-70	10.2	12-22-70	23.6
1-17-71	10.8	1-17-71	10.2

DISCUSSION

From the data presented, it can be seen that quantitative changes in nucleotides and RNA of the pulp of citrus fruit are gradual from approximately the mid-point of stage II until well into stage III of fruit development. An attempt will be made in this discussion to relate the concentration of total RNA and of certain RNA species and nucleotides to increases in fruit size, and to juice vesicle content of soluble solids, acids, and proteins.

Changes in concentration of total RNA were demonstrated to occur during fruit development ('Orlando' tangelo, 17 mg to 14 mg; 'Hamlin', 82 mg to 42 mg; 'Pineapple', 80 mg to 20 mg, 'Parson Brown', 101 mg to 10 mg). This was true whether total RNA was based on fresh weight or the AISR fraction from fruits. In the case of RNA concentration based on fresh weight, there was a decrease with time as the fruit was increasing in size. This basis of comparison does not compensate for an increase in the water content, and growth of the fruit pulp during stage II is primarily by cell enlargement, a process requiring increased water uptake (6). Thus, the apparent decrease in RNA could be the result of increased water content of the system. Indeed, when the increase in fruit volume became small, RNA content plateaued.

In the case of total RNA based on the AISR fraction, an increase occurred at about the same time there was a transition from stage II to stage III of fruit growth of 'Hamlin', 'Pineapple', and 'Parson Brown'. On the other hand, there was a slight decrease with 'Orlando'

tangelo. Three points can be made from these comparisons. First, fluctuation in water content of fruits probably would not affect the analyses greatly, and this seemed to be the case. There was no marked RNA change in fruits under water stress. Secondly, the quantity of AISR fraction in juice decreased as fruits enlarged. Yet, total RNA in the AISR fraction remained rather constant during this period. This could mean that total RNA was increasing, or at least was not being degraded as rapidly as other components in the AISR fraction. Thirdly, the apparent increase in RNA in the earlier part of stage III is probably due to the loss of pectins from the AISR fraction rather than an increase in total RNA in juice vesicles. As shown by Rouse et al. (87), pectin content in the AISR declines after the fruits have attained commercial maturity. Total pectin in the juice sac was found to decline from 12.55% in December to 9.55% in February.

The concentration of RNA in 'Pineapple' and 'Parson Brown' orange fruits decreased immediately following completion of stage II of growth. This may have been caused by environmental conditions. Both varieties experienced a prolonged drought and low temperature during this period, and increased ribonuclease activity has been shown to be associated with stress conditions (38, 55, 107). The other 2 varieties were in an irrigated grove which was heated during the November freeze.

Changes in the protein concentration during fruit growth and development were similar to those described for RNA. The protein concentration based on fresh weight was greatest in the earlier part of stage II; it decreased gradually with growth, reaching a plateau at completion of stage II. In the case of protein content based on the AISR fraction, an increase was observed at the time of transition from

stage II to stage III of fruit growth. A net increase in protein as a result of increased synthesis did appear to occur during this time since the protein concentration increased as the quantity of AISR decreased. However, part of this increase is possibly only apparent and is due to the loss of pectins from the AISR fraction. The constant level of protein occurring after completion of stage II probably indicates that protein in the fruit at this stage is less susceptible to degradation. Similar findings are reported by Spencer (96).

Additional information on the relation of RNA in the fruit during growth and development can be obtained from an analysis of the different species of RNA. Fractionation of RNA can be accomplished several ways; methylated albumen-kieselguhr (MAK) column, sucrose density gradient, and gel-electrophoresis. The latter method was chosen because of its quantitative reproducibility and its greater resolution of the various RNA species,particularly the ribosomal subunits and lower molecular weight RNAs.

Previous fractionation studies of RNA by other workers using gel-electrophoresis have indicated that RNA separated from green tissue is very different from the RNA separated from non-green tissue (69). Four major rRNA subunits have been identified in green tissue with sedimentation constants of 25S, 23S, 18S, and 16S. Non-green tissue contained only 2 major rRNA subunits with sedimentation constants of 25S and 18S. The 2 additional components in green tissue, 23S and 18S, have been designated as chloroplast rRNA subunits. The 25S and 18S species are subunits of cytoplasmic rRNA. With the use of the appropriate gel pore size, an additional RNA fraction having a much smaller sedimentation constant can be isolated. This fraction has been des-

ignated as sRNA and is comprised of several RNA species, i.e., 4S and 5S.

The use of gel-electrophoresis for the separation of RNA species from citrus fruit pulp tissue yielded excellent, reproducible fractionations. The addition of SDS (0.2% total concentration) to the extraction media and to the electrophoresis buffer was required to prevent any significant RNAase activity.

The electrophoretograms of both young and mature fruits were similar to those reported for green and non-green tissue by others. Two major RNA components were common to all fruits analyzed: the 25S and 18S subunits of cytoplasmic rRNA and sRNA. However, the electrophoretograms of young fruit differed from those of mature fruit in 2 respects. First, young fruit in the early part of stage II of growth contained 2 minor RNA components, Rf's 0.28 and 0.41, which were absent from the electrophoretograms of mature fruit; and secondly, the quantity of each component was greater in young fruit than in mature fruit. The 2 minor components are of particular interest because they correspond to the chloroplastic rRNA subunits reported by others (47, 69, 103). Yet, the fruit vesicles are devoid of chloroplasts; however, chromoplasts are present (89). Possibly, these 2 minor components are present in all plastids. The loss of these 2 components with growth is probably the result of degradation. Ingle (47), studying the synthesis and stability of chloroplastic rRNA, found that as the chloroplasts matured the percent degradation also increased. There was also a decrease in quantity of each RNA component with fruit growth of all 4 varieties. Studies by Ismail (50) and others (27, 78, 89, 91) have reported similar findings in other types of tissue, but they

reported an increase in the sRNA that accompanied the decrease in rRNA. This was especially noticeable in tissue during periods of rapid degradation of ribosomal RNA. However, it appears from this study that rRNA degradation does not contribute to sRNA.

Further analysis of the RNA species can be made by measuring the area under each peak and expressing the value as a ratio of the subunits for each rRNA. These ratios have been used by others to indicate the stability and turnover of rRNA (47, 103). The ratio of the cytoplasmic rRNA subunits, 25S and 18S, is usually 1.8-2.0 in most plant tissues (104). This is typical of cotyledons of seedlings 5 to 6 days after germination (103), and is often characteristic of plant tissue in a resting state (69). Increases in either rRNA synthesis or degradation or both, are thought to be reflected in ratios deviating from 1.8-2.0.

The 25S:18S ratio of the citrus fruit pulp increased up to the completion of stage II of fruit growth. Based on the study of Ingle (47), it would seem that the stability of the cytoplasmic rRNA of citrus increases up to the completion of stage II and remains stable well into stage III. This is further supported by the increase in the rRNA:sRNA ratio during the same period. Ismail (50) and others have found that with drastic declines in rRNA there was substantial increase in sRNA. The low ratios of 1.0 to 1.4 found in enlarging fruits (collected July 23, 1970, and September 26, 1970), is apparently the results of an active turnover in cytoplasmic rRNA. Increased cell division of the juice vesicle is occurring at this time (7).

The synthesis of RNA can be studied with the use of ^{32}P incorporated into RNA, separation of RNA by gel-electrophoresis, and detection of ^{32}P incorporation in the various RNA compounds. Studies of this

type with 'Calomondin' fruits yielded data similar to those reported by others (50, 68, 84). ^{32}P activity was found in the RNA components of the electrophoretograms of both young and mature, green fruit pulp, but absent in the electrophoretograms of very mature fruit prior to abscission. The amount of ^{32}P incorporated into the RNA of mature, green fruit was greater than that incorporated by young, enlarging fruit, indicating that the rate of RNA synthesis was greater in the former fruits. These data further support the increased RNA level found in the AISR fraction (Table 4) of the other varieties at completion of stage II of fruit growth since the mature green 'Calamondin' fruits probably correspond in development to the latter portion of stage II.

Particularly interesting was the amount of ^{32}P activity in the region of mRNA (RF 0.50) of the electrophoretogram of mature, green 'Calamondin' fruit. Several workers have designated this species as being a DNA-RNA complex (48, 49, 50). It is widely accepted that this DNA-RNA complex is an intermediate in the transcription of genetic information. It is suggested that synthesis of this complex in 'Calamondin', and possibly other citrus fruits, is one of the basic metabolic changes necessary to initiate fruit ripening and eventual senescence. Richmond and Biale (84) studying avocado fruit found that ^{32}P incorporation into rRNA and mRNA was high in the early phases of the climacteric, declined with the advent of the greatly increased utilization of O_2, and was absent at the maximum point of respiration. Frenkel et al. (37) have suggested that the normally coordinated cellular events of ripening may be initiated by a common denominator. The common denominator may be a substance that controls new RNA synthe-

sis necessary for all subsequent proteins synthesized and required for ripening. Ethylene has been found to hasten fruit ripening (21, 22) and also has been reported to cause a synthesis of the DNA-RNA complex (1, 2, 50). Thus these data support the idea of an increase in mRNA, and possibly protein synthesis just prior to the physiological events associated with senescence.

The electrophoretograms of very mature 'Calamondin' fruit prior to abscission showed an absence of distinct RNA components. This indicates much reduced synthesis of RNA. Similar data have been reported by Ismail (50).

It would seem that maintenance of RNA synthesis well into stage III is a very important characteristic which distinguishes citrus fruit, a non-climacteric fruit, from climacteric fruits. Several studies have indicated that degeneration of cells is accompanied by a failure to synthesize RNA (50, 63, 84). Ismail (50) was able to delay senescence in 'Calamondin' fruit peel and maintain synthesis of RNA with GA applications. Application of Kinetin, a senescence retardant, showed similar effects (62, 79, 97). Leopold (67) has suggested that senescence is regulated by RNA.

In contrast, ethylene treatments are reported to accelerate senescence in citrus fruits (39, 50) and other tissues (20), and cause a concommitant degradation of RNA (50). Recently Ku and Romani (63), using pear fruit, found that the onset of senescence was associated with a rapidly decreasing rate of ribosomal turnover, leading to a cessation of rRNA synthesis as the fruit reached the climacteric peak. In the same study, a distinct loss in polysomes occurred only in the post-climacteric phase. Similar findings for avocado fruits

are reported by Richmond and Biale (84). The burst of ethylene associated with ripening of climacteric fruits must greatly contribute to increased RNA metabolism observed in these fruits. It is interesting to note that removal of ethylene from tissue during the climacteric period, or preventing its production, delays ripening and the rate of cellular degradation (22).

The most important information gained from these data is that RNA in the fruit pulp does not undergo drastic changes such as observed during seed germination (27, 73) and during the climacteric phase of apples (70), pears (63), and avocadoes (84). Tissue culture studies of the juice vesicles by Bell (11) also support these findings. He observed that cells of the juice vesicles from mature and immature fruits, cultured in vitro differentiated into distinct juice vesicles and it was difficult to stimulate the tissues to produce callus-like growth.

Recently, several workers have reported isolating mononucleotides from various types of plant tissue. The identity of these nucleotides suggest that they are important intermediates in carbohydrate metabolism (18, 19, 90). In this study several nucleotides were isolated from the fruit pulp. Tentative identification was primarily based on the position of the component in the elution sequence from the ion-exchange column and also based on spectral analysis of the components. It is possible that several of the fractions were comprised of 2 or more nucleotides. This occurrence has been reported by others (90, 98, 106). Spectral analysis of these mixed fractions can indicate approximate quantity of nucleotides but further separation would be needed to establish identity. Also, the gradual elution of non-nucleotide

compounds at the beginning of gradient would alter the absorption spectrum of those nucleotides being eluted simultaneously. NAD is reported to be eluted from a formate anion-exchange column simultaneously with CMP (a) and AMP (b) (18). Also, the elution of GDP simultaneously with CTP and UDP simultaneously with ATP was found to occur in the standards. Peaks n and o (Fig. 31) could possibly be comprised of GDP-CTP and UDP-ATP, respectively. The identity of the fraction at position m is not known. Although, it was present in all samples analyzed and at a relatively high concentration. The only nucleotide positively identified and quantitated was ATP. Determinations of this nucleotide were made by the luciferin-luciferase procedure specific for ATP (24). The ATP concentrations in these various fruits are in agreement with those reported by Buslig (23) and would comprise less than 3% of the total concentration of peak o.

It is difficult to suggest particular roles for these compounds in the metabolism of the fruit pulp since conclusive identification has not been established. Yet, the quantity of these compounds found in the pulp and their tentative identification does suggest they are important intermediates in metabolism, particularly carbohydrate metabolism. NAD and the other nucleotides tentatively identified in these extracts are essential intermediates in both glycolysis and the organic acid cycle (29). Hassid (43) has discussed in detail the significance of nucleotides in the transformation of sugars in plants. Recently, Buslig (23) suggested that ATP is important in controlling acid accumulation in citrus fruit. ATP may be required for the translocation of acid from the mitochondria to the vacuole. A non-synchrony of synthesis of enzymes associated with the citric acid cycle gives

rise in the mitochondria to increased levels of citric acid. In the present study, the adenosine nucleotides were abundant in young fruits, coinciding with time of accumulation of acid in the vesicles, and declined to a very low level in mature fruits.

A number of studies have recently been made on nucleotides and their influence on modifying flavor (64, 65, 92). These studies indicate that the 5' mononucleotides were much stronger modifiers of flavor than the other derivatives. Of the 5' mononucleotides generally found in plant tissue, 5' GMP probably has the strongest effect. The minimum concentration of these compounds required to modify flavor is only several ppm. The concentration of several nucleotides in juice vesicles was in excess of this amount.

SUMMARY AND CONCLUSION

The quantity of nucleotides and RNA of citrus fruit during growth and development were studied in relation to increases in fruit size and to soluble solids, acidity, and proteins. Experimental material consisted of fruits of 'Orlando' tangelo, and 'Hamlin', 'Pineapple', and 'Parson Brown' oranges. It was concluded from this study that quantitative changes in RNA are gradual over the stages of fruit development examined. This conclusion was based on the following observations from total RNA and gel-electrophoretic determinations: Changes in concentration of total RNA were gradual from the mid-point of stage II until well into stage III, with only a slight increase just prior to completion of stage II of fruit growth. Gel-electrophoretic separation of RNA yielded patterns that indicated that RNA changes were gradual decreasing until the transition from stage II to stage III and then remained fairly constant for at least 2 months, and possibly longer. In 'Calamondin' fruit, maximum incorporation of ^{32}P into RNA occurred also at the transition from stage II to stage III, and appreciable ^{32}P activity was found in the mRNA region of the electrophoretogram. This component is believed to be a DNA-RNA complex. It was suggested that the increased RNA in the early part of stage III of fruit development may be essential for the initiation of changes associated with ripening of citrus fruit, e.g., production of enzymes required for changes normally associated with ripening.

Analysis of the acid-soluble nucleotide fraction of the fruit indicated that they are present in appreciable quantities in fruits and most surely play a vital role in fruit development. The nucleotides NAD, CMP, AMP, UMP, ADP, CTP, GDP, UDP, and ATP were tentatively identified based on the elution position from an anion-exchange column and spectral analysis. ATP was further assayed by the luciferin-luciferase method. The possible significance of nucleotides in carbohydrate metabolism and in the edible fruit was discussed, particularly as nucleotides may modify flavor.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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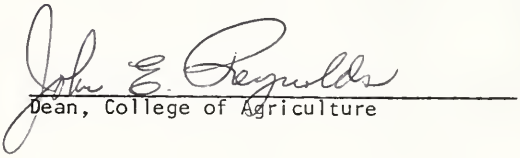
March, 1972



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